STRUCTURE AND FUNCTION OF THE SLIME SHEATH OF DICTYOSTELIUM DISCOIDEUM

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STRUCTURE AND FUNCTION OF THE SLIME SHEATH OF *DICTYOSTELIUM DISCOIDEUM*.

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ABBREVIATIONS USED

BSA	Bovine serum albumin.
ConA:	Concanavalin A.
DAB:	Diaminobenzidine.
ELISA:	Enzyme linked immunosorbent assay.
2-D electrophoresis:	Two dimensional gel electrophoresis.
FITC-antimouse:	Fluorescine isothiocyanate conjugated goat antimouse IgG (Fab'2 fragment).
HPLC:	High pressure liquid chromatography.
HRP-antimouse:	Horseradish peroxidase conjugated goat antimouse IgG (Fab'2 fragment).
kd:	Kilodaltons.
mAb:	Monoclonal antibody.
MW:	Apparent molecular weight estimated from mobility in SDS-PAGE.
SAC:	Formalin fixed cells of Staphylococcus aureas (Cowan strain).
SDS:	Sodium dodecyl sulphate.
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis performed in the discontinuous buffer system of Laemmli (1970).

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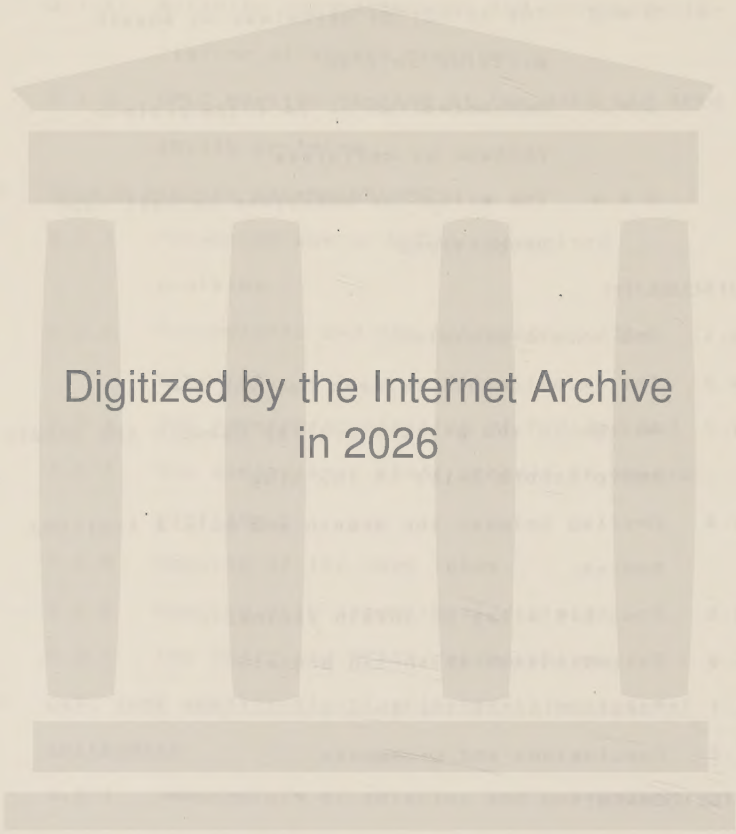
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ABSTRACT

The slime sheath of slugs of the cellular slime mould Dictyostelium discoideum has been proposed to be involved in several important aspects of this organism's differentiation. In the absence of detailed information concerning sheath composition, synthesis and structure, these proposals cannot be directly tested. The aim of this work was, therefore, to investigate the protein component of the sheath. With the aid of monoclonal antibodies, several sheath proteins were detected. Evidence is presented that a subset of these proteins are associated with sheath and stalk cellulose. Extensive sharing of antigenic determinants and proteins between the sheath, slug cells and mature fruiting bodies (but not vegetative amoebae) is shown. Much of the work described was greatly facilitated by the discovery of polymorphisms of one of the major sheath proteins. These polymorphisms probably map to a single gene on linkage group I. Some preliminary studies on the division of sheath function between its cellulose and protein components are also described. The significance of the results is discussed in terms of the structure, synthesis and function of the sheath.

CHAPTER 1 : INTRODUCTION

Numerous species of cellular slime moulds have been isolated from forest soil or humus, where they undoubtedly play an important role in the forest floor ecosystem (see Olive, 1975). All species so far described exhibit a basic asexual life cycle consisting of growth and division as free living, individual vegetative amoebae followed by differentiation to form a fruiting body. This consists of resistant spores supported by a stalk, generally composed of vacuolated non-viable cells, which aids the dispersal of the spores. The differentiation phase of the life cycle occurs when the food supply (usually bacteria) becomes limiting. When conditions are again favourable the spores germinate, giving rise to a new generation of vegetative amoebae.

In most cases the first stage of the differentiation phase is a starvation triggered aggregation of vegetative amoebae to form a multicellular organism. The final stage is the formation of the fruiting body. No cell fusion takes place during the multicellular stage of the life cycle.

One species of cellular slime mould, Dictyostelium discoideum (Raper, 1935), has been used extensively as a model system for the study of differentiation for a number of reasons:

(i) large numbers of cells can be easily grown, either in association with a bacterial food source or axenically in

semi-defined (Watts & Ashworth, 1970) or completely defined (Franke & Kessin, 1977) medium;

(ii) differentiation proceeds over a 24 h period in a well defined series of stages. Each stage is precisely timed, so that the large numbers of cells required for biochemical studies can be obtained for any given developmental stage;

(iii) the growth and differentiation stages of the life cycle are completely separate. Differentiation can, therefore, be studied without the added complication of concurrent cell division (Bonner & Frascella, 1952; Durston & Vork, 1978; but see also Zada-Hames & Ashworth, 1978). As a consequence of the separation of growth and development, mutants with impaired or altered development are able to grow normally, making them easy to isolate and maintain (eg. Williams & Newell, 1976);

(iv) the slug stage (see below) is composed of relatively constant proportions of partially differentiated cells and is amenable to molecular as well as more classical (eg cell graft) analysis (see MacWilliams, 1982).

1.1 The Asexual Life Cycle of D. discoideum

Detailed descriptions of slug biology and fruiting body formation have been published (Raper, 1940; Bonner & Eldredge, 1945; Raper & Fennell, 1952), and reviews of the major biochemical steps involved at each stage are available (Loomis, 1975 & 1982). The features of the life cycle most

pertinent to the work to be described here are:

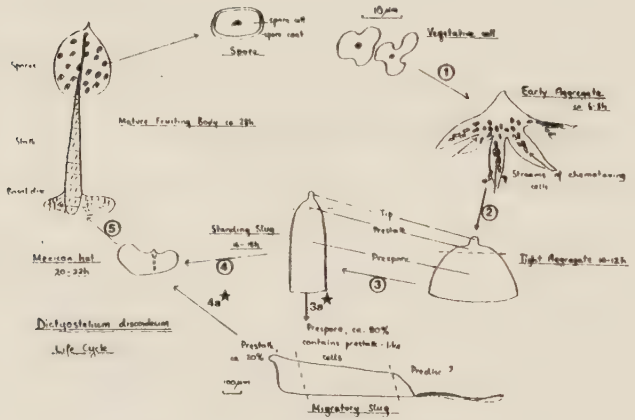


Figure 1.1: The asexual life cycle of D.discoideum.

Stage 1. Approximately 6 h after the initiation of starvation, vegetative amoebae begin to aggregate via chemotaxis to cyclic AMP (Konijn et al., 1967; Gerisch, 1982). The induction of specific developmentally regulated genes begins soon after starvation (eg. Alton & Lodish, 1977; Mehdy et al., 1983), presumably including the genes for components of the chemotactic apparatus (Devreotes, 1982) and specific cell-cell adhesion molecules (contact sites A, Beug et al., 1973). As the aggregate begins to form, it is covered by a thin cellulose-protein layer which is then synthesised throughout the entire developmental cycle until fruiting body formation (Shaffer, 1965; Treffry & Watts, 1978). This layer, the slime sheath, is the subject

of this thesis.

Stage 2. By the tight aggregate stage (10-12 h) the previously homogenous cell mass has differentiated to prestalk and prespore cells, which are spatially separated from each other in the aggregate and are the precursors of stalk and spore cells. These cells are not, however, irreversibly committed to a particular developmental fate (see Gregg & Karp, 1978; Morrissey et al., 1981). A morphologically distinguishable tip region (which has many of the properties of an embryonic organising region, Wolpert, 1971; Rubin & Robertson, 1975) also appears at this stage. Many changes in gene expression occur (Alton & Lodish, 1977).

Stages 3 & 4. After tight aggregate formation a "choice" must be made between the alternatives of immediate fruiting body formation (stage 4 in Fig. 1.1) or formation of a migratory slug (stage 3a in Fig. 1.1), whose assumed role is to move the aggregate to a more favourable environment for spore dispersal. The slug can re-enter the fruiting mode at any time (Stage 4a, see below).

Stage 5. The mature fruiting body is composed of a droplet of ca. 10^5 spores supported by a tapered stalk which is anchored to the substratum by a basal disc. Each spore consists of a quiescent amoeba encapsulated in a cellulose protein spore coat (Hohl & Hamamoto, 1969), while the stalk and disc cells are highly vacuolated and non-viable, with thick cellulose walls (Raper & Fennell, 1952).

1.2 The Slime Sheath

In general terms the sheath can be viewed in two ways.

Firstly, the sheath acts as a mechanical barrier between the cell mass and the outside world. Cells can neither enter nor leave through the sheath. Any metabolites which are to be excreted must leave via the sheath and any external stimuli must first pass through the sheath before they can be received. In this sense the sheath could act as a primitive homeostatic organ, influencing the out- and inflow of material and information.

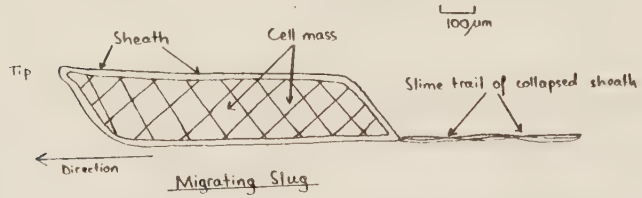


Figure 1.2: The migratory slug stage.

Secondly, the sheath provides the surface upon which morphogenetic cell movements occur and, in the migrating slug, it is the substratum over which slug cells migrate. During migration the slug can be viewed as a cohesive mass of cells moving through a pipe of their own making (the sheath, see Fig. 1.2) which is then left behind as a trail marking the slug's progress. In this sense the sheath is an extracellular matrix involved in morphogenetic movements and cell migration.

Extracellular matrices have been shown to play an important role in migration and morphogenesis in a variety of other, more complex, experimental systems, eg. sea urchin blastulation (Katow et al., 1982; McClay & Fink, 1982), chicken neural development (le Douarin, 1980) and mouse embryogenesis (Wartiovaara et al., 1980).

1.3 Proposed Roles of the Sheath in Development

In more specific terms, the proposed role(s) of the sheath can divided into three categories:

(a) The Slug-Fruit Switch: The "choice" between slug and fruiting body formation (Stages 3a & 4 Fig. 1.1) has been termed the slug-fruit switch and appears to be under the control of cells in the anterior of the slug (Smith & Williams, 1980). It is influenced by the environment (Newell et al., 1969; Bonner et al., 1982), genotype (Smith & Williams, 1980; Newell & Ross, 1982) and two metabolites (ammonia, high levels of which favour slug migration (Schindler & Sussman,1977), and a fruiting body inducing compound which is mimiced by propionate (Sussman et al., 1978)).

"Slugger" mutants with altered sensitivity to ammonia and a fruit inducing compound have been isolated (Sussman et al., 1978). However, other mutants with the same phenotype as these (ie. prolonged slug migration) but with normal responses to ammonia and propionate have also been isolated (Newell & Ross, 1982), implying that the slug-fruit switch

is more complex than simply ammonia and fruiter molecule.

The sheath has been shown to act as a barrier to the diffusion of some compounds into the slug (Farnsworth & Loomis, 1974) and it was proposed that it may also act as a barrier to the diffusion of ammonia out of the slug, thereby passively affecting the ammonia concentration and the slug-fruit switch (Schindler & Sussman, 1977).

(b) Pattern Formation and/or Maintenance: Prespore and prestalk cells can be easily distinguished using ultrastructural (Hohl & Hamamoto, 1969; Schaap et al., 1982), immunochemical (Takeuchi, 1963; Gregg et al., 1982; Tasaka et al., 1983), molecular (Gregg & Karp, 1978; Devine et al. 1982; Borth & Ratner, 1983) and enzymatic (Oohata & Takeuchi, 1977; Brown & Rutherford, 1980; Tsang & Bradbury, 1981) criteria. In many cases there is an obvious relationship between the partially and fully differentiated cells, eg. proteins which form part of the spore coat are expressed in (slug) prespore cells (Devine et al., 1983; Borth & Ratner, 1983), but the precise relationship between cells in the slug and the final fruiting body is unclear (see Morrissey, 1982).

How the correct proportions and spatial pattern of cell types in the aggregate are generated and maintained is also not known. Two types of mechanism have been proposed: the cell sorting and the positional models. In the cell sorting model, the cells first differentiate to prestalk and prespore in the correct proportions and then "sort out" to

give the correct prestalk/prespore pattern (Sternfeld & David, 1982; Meinhardt, 1983). In the positional model, cells differentiate according to their position in a hypothesised gradient of morphogen(s) so that proportion and pattern are established concurrently (Loomis, 1972; Krefft et al., 1983a).

In both cases diffusible molecules are probably required, so that if the sheath does act as a barrier to diffusion then a passive role for it analogous to that proposed in reference to the slug-fruit switch is possible. In particular, a tip to tail gradient of sheath thickness has been demonstrated (Farnsworth & Loomis, 1975) which could influence the gradient of morphogen proposed in the positional model.

An active role for the sheath has also been suggested (Ashworth, 1971) in which a proposed tip to tail gradient of sheath modification could directly provide positional information to cells. It is clear that some modification of the sheath does occur *in vivo* (Watts & Treffry, 1975) but it is not known what function this modification has.

(c) Slug Migration: The nature of the substratum is important in cell-substratum interactions (Gingell & Vince, 1982). Since the sheath is the substratum over which slugs migrate, it must at least allow and probably facilitates cell migration.

In addition to providing the substratum over which the

slug cells migrate, the sheath plays a physical role in maintaining the polarity of migration because only at the tip is it fluid enough to be deformed by slug cells (Francis, 1962; Shaffer, 1965; Loomis, 1972). Despite its relative fluidity at the tip, the sheath still presents a significant resistance barrier to slug migration and may play a role in determining the speed of slug migration (Inouye & Takeuchi, 1979; see Appendix).

1.4 The Composition of the Sheath

The sheath is composed of randomly oriented cellulose fibrils of 10-15nm diameter embedded in an amorphous protein matrix (Hohl & Jehli, 1973).

A urea-SDS insoluble component of the sheath has been isolated and extensively characterised (Freeze & Loomis, 1977a). It was composed of 60% cellulose, 15% protein, 3% heteropolysaccharide, 5% lipid and 1% sulphate. The crystallinity of the cellulose component was proposed to play an important role in determining the strength of the sheath. The cellulose of non-migratory stages (eg tight aggregates) was less crystalline than that of migratory slugs (Freeze & Loomis, 1977a) and mutants which lack the enzyme N-acetylglucosaminidase (Dimond et al., 1973) also had cellulose of lower crystallinity (Freeze & Loomis, 1977b). Slugs of these strains break up during migration (Dimond et al., 1973), allegedly due to the lower strength of their sheath cellulose (Freeze & Loomis, 1977b). It is unclear, however, how N-acetylglucosaminidase affects

cellulose crystallinity since (a) cellulose contains little or no N-acetylglucosamine and (b) the sheath associated N-acetylglucosaminidase acts on protein, not cellulose (Watts & Treffry, 1975).

Despite this uncertainty, the cellulose component of the sheath is possibly important for slug migration because UDPG-pyrophosphorylase mutants, which are unable to make cellulose, fail to migrate (Dimond et al., 1978). The aggregates of these mutants appear reasonably normal up to slug formation and are covered by a sheath, presumably composed of the non-cellulose (protein ?) component (Freeze & Loomis, 1977a & b).

Little is known about the protein component of the sheath. The protein fraction of the urea-SDS insoluble material isolated by Freeze and Loomis (1977a) was composed of a disperse range of peptides of 1,000-15,000 Daltons which were apparently covalently linked to cellulose. In the only published study of sheath proteins, Smith and Williams (1979) showed that whole sheath is highly proteinaceous (ca. 60% protein) and that at least 12 proteins spanning a wide range of molecular weight (>100,000 to ca. 15,000 Daltons) could be released from the sheath by mild SDS treatment.

1.5 Experimental Approaches to Sheath Biochemistry

The work of Freeze and Loomis (1977a & b) and Smith and Williams (1979) differed in two major aspects.

Firstly, the use of urea-SDS before analysis (Freeze &

Loomis, 1977a) would have certainly removed the proteins reported by Smith and Williams (1979) and explains the discrepancy between the two reports.

Secondly, Freeze and Loomis (1977a) analysed the sheath which actually covers the slug, so that pre-treatment with urea-SDS was necessary to remove all traces of slug cells. Smith and Williams (1979) analysed the slime trails ie. the collapsed sheath left behind during slug migration (see Fig. 1.2). This material can be obtained virtually free from cellular contamination and can, therefore, be analysed without pre-treatment.

One is faced, therefore, with the problem of either examining the sheath which covers the slug but having to remove the bulk of the protein from it before analysis or examining untreated, whole material which has been left behind by the slug. The work described in this thesis was carried out using the latter approach of examining the slime trails and the terms "sheath" and "slime trail" are used interchangeably. This assumes that the sheath and the slime trails are essentially the same.

The choice of slime trails was largely governed by the view that the protein component of the sheath, which cannot be studied using the approach of Freeze and Loomis (1977a), is potentially the most interesting. For example, the anterior-posterior gradient of sheath thickness is due to the continual deposition of the non-cellulose, presumably protein, component along the entire length of the slug

(Farnsworth & Loomis, 1975). In terms of sheath modification (Ashworth, 1971; Loomis, 1972; Watts & Treffry, 1975) proteins can be modified in a variety of ways (glycosylation, phosphorylation, sulphation, site specific cleavage) which could conceivably convey information.

In view of the lack of information about any sheath protein, a means was sought to obtain specific molecular probes for some or all sheath proteins. Monoclonal antibodies (Kohler & Milstein, 1975) provide such probes.

1.6 Monoclonal Antibodies

Monoclonal antibodies directed against uncharacterised, unknown antigens can be obtained via the fusion of spleen cells (B-lymphocytes) from an appropriately immunised mouse with a mouse myeloma cell line, followed by selection for the desired hybridoma (spleen x myeloma) cells (see Kennett et al., 1980).

The selection procedure is in two stages. Hybridoma cells are first selected on the basis of growth in HAT medium (which contains Hypoxanthine, Aminopterin, Thymidine). Aminopterin inhibits dihydrofolate reductase (see Fig. 1.3) so that *de novo* nucleotide synthesis is blocked and the salvage pathways, utilising hypoxanthine for purine biosynthesis and thymidine for pyrimidine, must be used. The myeloma cells lack the enzyme hypoxanthinephosphoribosyl transferase (HGPRT), so they cannot synthesize purines in the presence of aminopterin and die in the HAT medium.

Spleen cells are unable to grow in any tissue culture medium. The hybridoma cells have a functional HGPRT (from the spleen cell) and are able to grow in tissue culture (a myeloma cell characteristic). Consequently, only hybridoma cells are able to grow in the HAT medium; all other cells die.

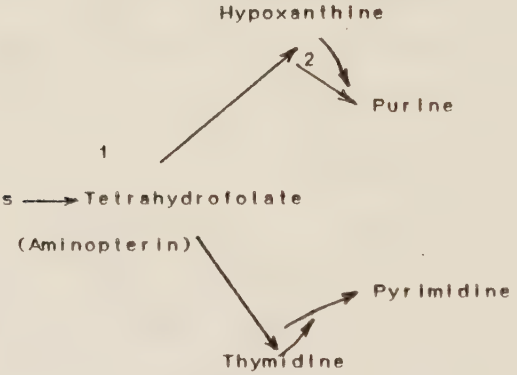


Figure 1.3: The basis of HAT selection. Enzymes: (1) dihydrofolate reductase, (2) hypoxanthinephosphoribosyl transferase (HGPRT). Aminopterin blocks at (1) and the myeloma cells lack (2) so they cannot grow in the presence of aminopterin.

The second selection step involves screening the hybridoma cells for production of the relevant antibody and is described in detail in Chapter 2.

Monoclonal antibodies have two great advantages over conventional antisera. Each hybridoma secretes a single

(monoclonal) antibody which is directed against a single specific antigenic determinant ie they are reagents are great specificity. Once obtained, a hybridoma is essentially immortal. It is possible, therefore, to produce large quantities of a highly specific reagent without the problem of batch to batch variability often encountered with conventional antisera. These antibodies can then be used to both purify and analyse any molecule they recognise, including molecules whose existence and characteristics were previously unknown.

1.7 Aims of the Work

The principal aim of this work was to resolve the uncertainty concerning the presence of protein in the sheath and to determine whether any sheath associated proteins are sheath specific. It was also hoped that characterisation of sheath proteins would provide a basis for studies of the role(s) of the sheath and its relationship to other "tissues" during *D.discoideum* differentiation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

ConA, horseradish peroxidase (RZ 1.6), DAB, tunicamycin, leupeptin and papain were from Sigma. Cellulase (EC 3.2.1.4, 20mU/ mg from Basidiomycetes) and Pronase were from Merck. HRP-antimouse and FITC-antimouse (Fab'2 fragments) were from TAGO Immunodiagnostics, U.S.A. Nitrocellulose filters (0.45μm pore size) were from Schleicher and Schuell. Titertek ELISA 96 well plates were from Linbro. Trypsin (TPCK treated) was from Worthington.

The HPLC column used for peptide mapping was 8 x 300mm, with a 8 x 25mm precolumn, both packed with Shandon ODS Hypersil (5μm diameter beads), a C_{18} reversed phase support. Protein-A Sepharose, CNBr-activated Sepharose and Sephadex G-50 were from Pharmacia. SAC was from Calbiochem and Bio Gel P-6 from BioRad.

Acrylamide (twice recrystalised) for electrophoresis was from Serva and Ampholines were from LKB. Molecular weight standards (phosphorylase B, 92.5kd; BSA, 66.2kd; ovalbumin, 43kd; carbonic anhydrase, 31kd; soybean trypsin inhibitor, 21.5kd and lysozyme, 14.4kd) were obtained as a kit from BioRad.

For Dictyostelium culture, Oxoid yeast extract L21 and bacteriological peptone L37 and Difco Bacto Agar and, for particular experiments (see below), Difco low ash Noble agar were used. For tissue culture, all media except for the foetal calf serum, were from Gibco (RPMI 1640 was used).

Foetal calf serum was from BioLabs.

2.2 Culture Conditions and Strains

Unless otherwise noted, all work described here was performed using the haploid strain NP84, a derivative of the V12 isolate (North & Williams, 1978). Other haploid strains used were NC4-Raper, Dd61, WS380B, WS472, WS576 and WS584, (all of which are non-mutagenised wild isolates of *D. discoideum*), NP73 (the parent of NP84) and HU1628 (an NC4 derivative in which 6 of the 7 linkage groups are genetically marked). The diploid strain DP62 (the isogenic diploid of NP73, Stenhouse & Williams, 1981) was also used. Other diploid strains which were constructed as a part of this work are described in the Results section.

Standard culture conditions were used for the growth of amoebae and the preparation of migrating slugs (Smith & Williams,1979 & 1981). Briefly, vegetative amoebae were grown on nutrient agar with *Klebsiella aerogenes* as the food source at 21 $\pm 1^{o}$C, washed free of bacteria and then plated at high cell density ($>10^{7}$ cells/cm^{2}) on non-nutrient agar plates, generally in a 5 x 1cm strip at one side of the plate. The plate was then placed in an opaque PVC container with a small hole in one side to provide a lateral light source. Under these conditions (ie. low ionic strength, lateral light) the strains used here do not form fruiting bodies, but rather form slugs which migrate towards the light source.

2.3 Preparation of Sheath and Cellular Extracts

Sheath was prepared by allowing the slugs to migrate

across the Petri plate towards the light source for 6-7 days. By this time virtually all the slugs had completely traversed the plate, leaving it covered with a layer of sheath left by the slugs as slime trails as they migrated. This material is virtually free from contaminating cells (Smith & Williams, 1979). To obtain pure sheath it is essential to use strains which do not leave cells in their trails as they migrate. For this reason, NP84 was chosen (Smith & Williams, 1979). Sheath was scraped from the plates into distilled water, thoroughly homogenised and then washed by centrifugation 3 times (1000g, 5 min.).Following washing the sheath was either used immediately or lyophilised and stored dessicated at 4°C. This procedure generally yielded 100-200μg sheath per 10^8 cells.

In some experiments, the trails from individually transferred slugs (see Appendix) which had been migrating for 18-20 h were collected rather than sheath prepared from mass cultures as described above. In particular, where various compounds were tested for their effect on the sheath, slugs were transferred to small Petri plates (50mm diameter rather than 85mm, containing 5ml Noble agar rather than 30ml of standard Bacto agar) containing the compound of interest included in the agar. In these cases an SDS extract of the trails of 10-20 slugs (ca. 20cm of sheath) was sufficient for one electrophoresis lane.

Proteins were extracted from the sheath in either freshly made 8.0M urea, followed by dialysis against the desired buffer or desalting over a BioGel P-6 column with subsequent concentration by ultrafiltration (Millipore PTGC

ultrafiltration membrane, nominal MW cut-off 10kd), or in Tris-SDS buffer (1% SDS in 125mM Tris-HCl, pH 6.8). Release of a subset of sheath proteins was also achieved by mild cellulase enzyme digestion of the sheath with 0.25mg/ml (w/v) cellulase in 0.1M sodium acetate buffer, pH 4.5. In all cases the sheath concentration was 5mg/ml (w/v) and extractions were for 60 min. at 40 ^{o}C for urea and SDS, or 2 h at 40^{o}C for cellulase. For 2-dimensional gel analysis, extraction was carried out at 20^{o}C in the lysis buffer described by O'Farrell (1975), which contains 9.5M urea. Insoluble material was removed by centrifugation for 10 min. in an Eppendorf centrifuge.

Spore cell and coat extracts were prepared according to Wilkinson and Hames (1983). Before extraction the spores were extensively washed in distilled water and filtered through Nitex mesh to remove contaminaing stalks (the mesh allowed the spores to pass through and retained the stalks). Spore purity was monitored microscopically to ensure no contamination with stalk cells had occurred. The stalk cells retained in the mesh were extracted with Tris-SDS to yield a stalk extract.

Proteins were extracted from vegetative and slug cells in Tris-SDS at 5 x 10^{6} cells/ml. In some cases, slug cells were disaggregated by mild papain treatment prior to extraction (Krefft et. al, 1983b). It was found that 10^{4} to 5 x 10^{4} cells were sufficient for one electrophoresis lane.

Proteins secreted by slug cells in suspension were obtained by dissociating slug cells with papain, washing the single cells 3 times with MES-PDF buffer (Landfear &

Lodish,1980) and resuspending them at 5 x 10^6 cells/ml in the same buffer. The cell suspensions were shaken at 180 rpm at 21±1°C for various times (see Results). Protein secretion was analysed by SDS-PAGE and by a competition ELISA (see below).

When required, protein concentrations were determined using a commercially available version of the Coomassie Blue dye binding assay (BioRad).

2.4 Preparation of Monoclonal Antibodies

Female Balb/c mice (6-8 weeks old) were injected intraperitoneally with 100-150µg of urea extracted sheath proteins (of strain NP84) in complete Freunds adjuvant, followed by a second identical immunisation 4-6 weeks later. Three days before fusion the mice were boosted intravenously with 100µg of urea extracted sheath proteins in normal saline (0.9% NaCl, w/v). For each fusion the spleen cells from two mice were pooled and the number of spleen cells per fusion kept constant at 10^7, with 10^7-2×10^7 mouse myeloma cells (Balb/c X63-Ag8.653, a mutant myeloma cell line which does not produce immunoglobulin chains of its own, Kearney et. al., 1979). Fusion and subsequent cloning were performed according to de St. Groth and Scheidegger (1980).

Hybrids were screened for anti-sheath activity by an ELISA. Urea extracted sheath proteins were coupled to 96 well ELISA plates at approximately 1µg/ml, 50µl/well, in carbonate-bicarbonate buffer, pH 9.6 and the plates washed and stored according to Voller et. al. (1979). For assay, 50µl of medium from cultures containing growing hybrids were

added to duplicate wells of the ELISA plate, incubated for 1-2 h at 37°C (or overnight at 4°C) then washed 3 times with PBS-T (phosphate buffered saline; 9.0g NaCl, 0.2g KH_2PO_4, 2.9g Na_2HPO_4, 2.0g KCl, pH 7.2, containing 0.05% Tween-20, w/v). Then 50µl of HRP-antimouse (diluted 1/3000 in PBS-T) was added to each well and incubated for 1 h at 37°C, followed by 3 further PBS-T washes. Antibody was detected using orthophenylenediamine as the substrate (4mg/ml in citrate-phosphate buffer, pH 5.0, containing 0.0015% H_2O_2) and the reaction was then stopped by the addition of H_2SO_4 to a final concentration of 0.5M (Volter et al., 1979) when positive controls had reached an absorbance of ca. 1.0 unit. Absorbances were read using a MicroELISA reader at 488nm. Background absorbance was generally 0.01-0.02 units and wells with an absorbance of >0.1 units were considered to be positive. The HRP-antimouse antibody used was directed against mouse IgG, so only those hybrids secreting IgG were detected.

Cultures were screened as soon as obviously growing cells were visible and subsequently cloned as soon as cells reached half confluence in the tissue culture wells. Cloning at limiting dilution was carried out according to de St Groth and Scheidegger (1980) at least 3 times before the hybrids were used. Growth of hybrids as ascites tumours in mice and the purification of IgG by protein-A affinity chromatography were according to Goding (1980).

2.5 Determination of the Nature of the Antigenic Site

To determine whether the mAb's recognised the peptide or carbohydrate portion of glycoproteins, a modification of the

ELISA was developed (the competition ELISA). A glycopeptide fraction of urea extracted sheath proteins was prepared by exhaustive Pronase digestion (Spiro, 1972). Various amounts of either undigested protein or an equivalent amount of the glycopeptide fraction were added to duplicate wells of the ELISA plate at the same time as a constant amount of purified IgG of the mAb to be tested ie. if 1µg of undigested protein was added then an amount of the glycopeptide fraction which would have contained 1µg of protein before Pronase digestion was added to the corresponding glycopeptide well of the plate.The added antigens in solution compete for antibody with those bound to the plate, thereby decreasing the amount of antibody bound to the plate and hence the final absorbance (since the added, soluble antigens and the antibody which has reacted with them are washed away, see 2.4). If an antigenic determinant is carbohydrate, the undigested protein and the glycopeptide fractions should both inhibit the ELISA to the same extent. If the determinant is protein, the glycopeptide fraction should not inhibit the ELISA at all.

In some experiments the glycopeptide fraction was fractionated by gel filtration on a Sephadex G-50 column (column dimensions, 1 x 90cm) in 0.1M pyridine acetate buffer (pH 5.0, Spiro, 1972). After lyophilisation, fractions were dissolved in PBS-T and tested at an appropriate dilution in the competition ELISA.

2.6 Electrophoretic Conditions

Discontinuous SDS-PAGE was performed using the buffer system of Laemmli (1970) in 1mm thick slab gels (resolving

gels were of varying concentrations with a 4% acrylamide stacking gel). Gels were run at 7.5mA for one hour and then 17.5mA until the tracking dye was 1-2cm from the end of the gel. Samples were of ca. 20μl.

Two-dimensional gel electrophoresis was carried out exactly according to O'Farrell (1975), using a pH 4-8 range in the first dimension.

Staining of gels for protein was carried out with Coomassie Blue (essentially according to Fairbanks et al.,1971) or using a simplified silver staining procedure (Morrissey, 1981).

For the staining of ConA binding glycoproteins, a modification of the ConA-peroxidase technique (Wood & Sarinana, 1975) was developed. Following, SDS-PAGE proteins were electrophoretically transferred from the acrylamide gel to a nitrocellulose filter (Towbin et al.,1979). The filter was then washed for 40 min. in 4 changes (10 min. per wash) of Tris-NaCl buffer (0.8% NaCl, 10mM Tris-HCl, pH 7.4) and incubated overnight at room temperature in a moist chamber or sealed plastic bag with 20μg/ml ConA in Tris-NaCl buffer containing 3% w/v BSA and 2% w/v sodium azide. The filters were then washed for 4 x 10 min. in Tris-NaCl buffer and further incubated with 100μg/ml horseradish peroxidase in Tris-NaCl buffer containing 3% BSA. Following 4 further washes with Tris-NaCl buffer,ConA binding proteins were visualised by incubation of the filters with DAB (in Na_2HPO_4/NaH_2PO_4 buffer, 15mM with respect to phosphate, pH6.5, containing 0.5mg/ml DAB and 0.0015% H_2O_2).

Immunoblotting of electrophoretically separated proteins (to identify the proteins reacting with each of the mAb's) was identical to the ConA-peroxidase staining except that purified mAb IgG at 0.2-0.5μg/ml replaced ConA in the first step and HRP-antimouse (diluted 1 in 2000) replaced peroxidase in the second step. For both ConA-peroxidase staining and immunoblotting, molecular weight standards were also transferred to nitrocellulose and the lane containing them stained separately with Amido Black. The Amido Black stained filters shrank to varying degrees during the staining procedure, which made precise estimates of molecular weight for transferred proteins impossible. Consequently, molecular weight estimates for transferred proteins were accurate to approximately ±1000 Daltons.

2.7 Monoclonal Antibody Affinity Chromatography of Sheath Proteins

For preparative purposes, mAb affinity colomns were made using MUD50 and MUD51 coupled to CNBr-activated Sepharose. Following the manufacturer's instructions, 10mg of purified IgG (obtained from ascitic fluid) was coupled to 5ml (wet volume) of CNBr-activated Sepharose. Total coupling time was 4 h, after which greater than 90% of the IgG was coupled (as estimated by ELISA of the antibody solution after coupling). These columns were used several times with no apparent loss in antigen binding capacity. Columns were stored and run at 4°C.

The procedure used for running the columns was based on that of McMaster and Williams (1979). Urea extracted sheath proteins were dialysed into column running buffer (0.5M

NaCl, 10mM Tris-HCl, 0.05% Tween 20, 0.1% sodium azide, pH 8.0) and the protein concentration and the antigen content determined. The protein solution was then diluted to ca. one column volume and run into the column at 0.05ml/min, followed by washing in the same buffer until the OD_{280} had returned to the baseline. Bound proteins were eluted in 50mM diethlyamine (pH 11.5) then immediately neutralised with solid glycine and dialysed against Tris-NaCl buffer. The column was washed with several column volumes of running buffer to remove all traces of elution buffer and preserve antibody activity.

Following dialysis, the protein and antigen content of the bound and unbound fractions were determined again. In general, the recovery of protein and antigenic activity from the columns was greater than 90% and no antigenic activity could be detected in the unbound fraction at column loadings of up to 300μg. Material from the columns was stored in aliquots at -20^{o}C. As the protein concentration of the fractions was often low, samples for electrophoresis were precipitated with 4 volumes of cold (-20^{o}C) acetone for 3-4 h or overnight at -20^{o}C before being dissolved in sample buffer.

For analytical purposes, antigens were immunoprecipitated using SAC (Kessler, 1975). Although mouse IgG is not always efficiently bound by protein A (Lindmark *et al.*, 1983) it was found that all of the mAb's used here could be purified by protein A affinity chromatography and could be used for SAC immunoprecipitation without a second antibody. The SAC capacity for each antibody was determined prior to use by

incubation of a known amount of IgG with increasing amounts of SAC (60 min. on ice) then measuring the antibody activity of the supernatant following centrifugation (5 min. in an Eppendorf centrifuge). The binding capacity of SAC for all the mAb's used was in the range 5-10μg IgG bound/μg SAC. The immunosorbent was always used in at least twofold excess over antibody added.

For immunoprecipitation, urea extracted sheath proteins were dialysed against NET-NP40 (Kessler, 1975) and divided into aliquots. Each aliquot (100-200μl, containing 5-10μg protein) was incubated for 30 min. on ice with 25μl of a 10% w/v suspension of SAC to remove any non-specifically binding material, followed by centrifugation (3 min. in an Eppendorf centrifuge). The supernatant was then made 1.0mg/ml with respect to BSA and incubated with 0.5-1.0μg of mAb IgG for 2 h on ice, then 25μl of SAC suspension was added and the incubation continued a further 60 min. The SAC pellet was collected by centrifugation, washed 3 times with 1.0ml of NET-NP40 then taken up in an appropriate volume of electrophoresis sample buffer. In all cases, control incubations with no antibody (to monitor non-specific SAC precipitation) or with no antigen (to ascertain the position of SAC and/or antibody derived bands) were performed. Pre-treatment of the SAC with SDS-βmercaptoethanol (Richert et al., 1979) was found to be necessary to give low background levels of staining on immunoblots.

2.8 Determination of mAb Cell Type Specificity

Three methods were used to determine the cell type specificity of the mAb's:

(a) the anterior 10-15% (prestalk) and the posterior 85-90% (prespore plus some prestalk-like cells, see Introduction) portion of slugs were separated by microsurgery and separately immunoblotted. This is a relatively crude method which does not differentiate between intra- and extracellular proteins

(b) 5μm frozen thin sections of slugs (prepared by Prof. J. Gregg) were stained with each mAb, using FITC-antimouse as the second antibody (Gregg et al., 1982). This allows *in situ* localisation of the antigens within the slug to specific cell types.

(c) Flow cytometry using a FACS-IV cell sorter was performed according to Krefft et al. (1983b) with data analysis according to Voet et al. (1983). FACS analysis provides an accurate quantitative measure of the proportion of cells in a population which carry a given antigen on their cell surface.

2.9 Peptide Mapping of Sheath Proteins

Sheath proteins of NP84 which were bound by the MUD50-Sepharose column were peptide mapped by a procedure based on that of Elder et al. (1977) using reversed phase HPLC rather than thin layer techniques for peptide resolution.

Following concentration by acetone precipitation, 10μg of MUD50 bound sheath proteins were separated by electrophoresis on a 10-20% SDS-acrylamide gradient gel, stained for 45 min. with Coomassie Blue R (0.05% Coomassie Brilliant Blue R in 25% isopropanol/10% acetic acid) and destained for 90

min. in 25% isopropanol/10% acetic acid followed by overnight washing in 25% isopropanol to complete destaining and remove SDS. Stained bands were cut from the gel and washed in several changes of 15% methanol, then lyophilised.

The lyophilised individual gel slices were radioiodinated using the Bolton-Hunter reagent (Bolton & Hunter, 1973; purchased as the mono-iodide, 2200 Ci/mmole, from New England Nuclear) at 25μCi per gel slice (Kelly et al.,1981). After labelling, each gel slice was washed for 24 h in several changes of 15% methanol and then re-lyophilised and counted. Depending on the amount of protein present, specific activities of 1-2 x 10^6 cpm/μg protein were generally obtained. The radiolabelled gel slices were rehydrated in 0.5ml of 50mM $(NH_4)_2CO_3$, pH 8.0, and homogenised. A sample of ca. 10^4 cpm was withdrawn for immunoblotting and autoradiography. No changes in antibody reactivity or MW of any of the labelled proteins was seen (data not shown). The remaining protein was digested overnight with 25μg of TLCK-treated trypsin (at 37°C). After digestion, the slurry was filtered through glass wool and the filtrate, containing released tryptic peptides, was lyophilised and counted.

For HPLC, the tryptic peptides were dissolved in buffer A (0.1% trifluoroacetic acid in H_2O) at 10^6 cpm/ml and 100 μl or 10^5 cpm applied for each column run. Elution was carried out in a discontinuous gradient of buffer B (0.1% trifluoroacetic acid in acetonitrile) with a flow rate of 0.7 ml/min. The gradient was:

t = 0 to 2 min., 5% B, no gradient

t = 2 to 9.5 min., 5% to 20% B, gradient = +2% B/min.

t = 9.5 to 92.5 min., 20% to 61.5% B, gradient = +0.5% B /min.

t = 92.5 to 100 min., 95% B, no gradient.

Fractions were collected every 0.5 min. (ie. 0.35 ml/fraction) and counted. An internal standard was provided by monitoring the elution of the auto-digestion peptides of the trypsin at 280nm. In addition to this, two controls were performed. Firstly, a slice of gel containing no Coomassie Blue stained material was treated in parallel to the stained slices to determine the non-specific level of ^{125}I incorporation. Secondly, the ability of the chosen conditions to resolve the tryptic peptides of a group of standard proteins in the same MW range as the sheath proteins to be mapped was tested by preparing HPLC maps of ^{125}I peptides of BSA, ovalbumin and carbonic anhydrase (ie. the SDS-PAGE MW markers).

The HPLC peptide maps of deglycosylated versus normally glycosylated proteins were compared. Initially deglycosylation in the gel slice using HF (Mort & Lamport, 1977) was attempted but the recovery of material from the HF-treated gel slice was too low to allow HPLC analysis. As an alternative, deglycosylation with HF was performed on the tryptic peptides (ie. after trypsin digestion). In both cases the lyophilised gel slice or tryptic peptides were treated with ca. 100μl of 100% HF in a teflon tube at $0^{o}C$ for 30 min. The HF was removed by evaporation in a gentle stream of dry N_2 and the sample lyophilised and treated as above for HPLC.

2.10 Genetic Analysis of Sheath Protein Polymorphisms

The method used for the mapping of the *psp*A locus is described in detail in Chapter 3.2.

CHAPTER 3: RESULTS

CHAPTER 3.1: BASIC CHARACTERISATION OF SHEATH PROTEINS AND ANTI-SHEATH MONOCLONAL ANTIBODIES

3.1.1 Release of Sheath Associated Proteins by Denaturing Agents

Proteins can be released from the sheath by protein denaturing agents. A comparison using SDS-PAGE of proteins released by SDS extraction of the sheath and of slug cells shows quantitative and qualitative differences in their protein complements (Fig. 3.1.1a, lanes 1-3). This clearly demonstrates that the sheath contains a large number of proteins in addition to the principally non-protein urea-SDS insoluble fraction described by Freeze and Loomis (1977a, see Chapt. 1). Many cellular proteins are reduced in or absent from the sheath while others are apparently present in large amounts. There are a number of apparently sheath specific proteins (arrows, Fig. 3.1.1a, lane 3).

SDS-PAGE comparison of ConA binding sheath and slug cell glycoproteins also shows quantitative and qualitative differences between the two (Fig. 3.1.1b). Proteins which stain only in sheath extracts are indicated by arrows (Fig. 3.1.1b, lane 3).

Different staining techniques were used in Figs. 3.1.1a & b, making direct comparison between them impossible. However, both show that the sheath contains a large number

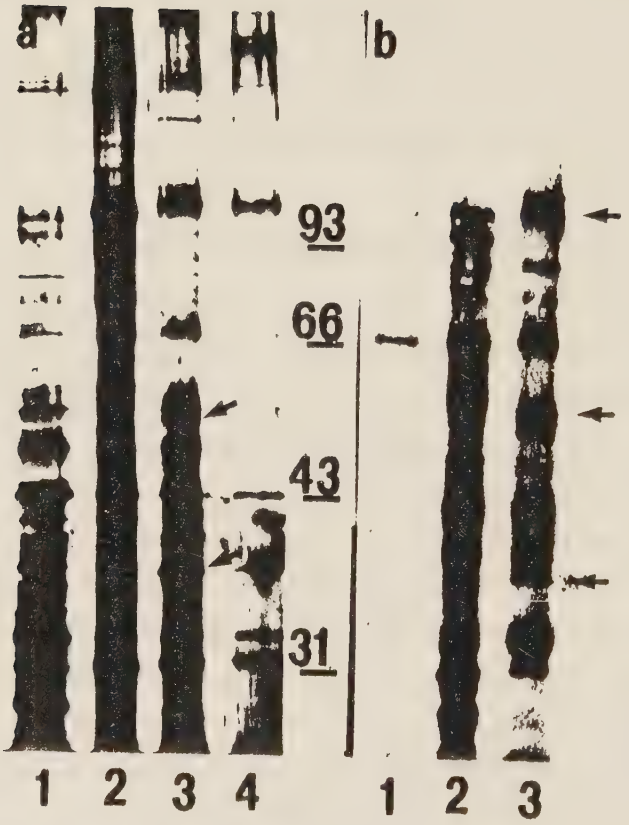


Figure 3.1.1: SDS-PAGE of slug cell and sheath proteins. (a) Silver staining (b) ConA-peroxidase staining. Lanes: (1) slug cells; (2) slug cell crude membranes; (3) sheath; (4) sheath from individually transferred slugs. 10% acrylamide resolving gel.

of proteins, some of which may be sheath specific and others which may be due either to contamination of the sheath with slug cells and/or the sharing of proteins between the sheath and slug cells. One dimensional SDS-PAGE does not allow resolution of proteins which may be genuinely common to both or simply have very similar MW.

To decrease the possibility of cellular contamination, the sheath from young individually transferred slugs of strain NP84 was collected and extracted. In this case, the extraction was performed directly in SDS-PAGE sample buffer, which contains β-mercaptoethanol in addition to SDS. Comparison of sheath prepared in this way with the standard sheath preparation (Fig. 3.1.1a, lanes 3 & 4) shows that a number of bands are missing from the sheath extract of transferred slugs. Although this may be partly due to differences in the amount of protein loaded, it suggests that the standard preparation may be slightly contaminated with cellular proteins. However, many bands with the same mobility remain eg. a major sheath protein at ca. 32kd has a cellular counterpart. With the exception of a high MW band which barely enters the gel, no additional proteins are seen in the sheath from transferred slugs despite the addition of β-mercaptoethanol.

Urea (8.0M) and guanidine-HCl (6.0M) also release proteins from the sheath (data not shown). Release by these agents is less efficient than by SDS but qualitatively identical. Non-denaturing detergents (eg Triton X-100) did not detectably release sheath proteins.

3.1.2 Cellulase Enzyme Release of Sheath Proteins

Given that the sheath is a complex of cellulose and protein (Hohl & Jehli, 1973), degradation of the sheath's cellulose component should release any proteins which are retained in the sheath by interactions with cellulose.

Figure 3.1.2 shows that such cellulase released proteins do exist. Comparison of lane 5 (sheath alone), lane 4 (enzyme alone) and lanes 1-3 (sheath + enzyme) in Fig. 3.1.2a shows that at least two proteins (of ca. 32kd and 40kd, arrows) are released in a time and enzyme dependent manner. This protein release was accompanied by the release of glucose into the incubation mixture, presumably from the degradation of cellulose.

The protein release is seen more clearly in Fig. 3.1.2b, where the same material as shown in Fig. 3.1.2a was immunoblotted and stained with a mAb which recognises a large number of sheath proteins (see 3.1.3). In addition to the 32kd and 40kd proteins shown by silver staining, several other higher MW sheath proteins were released by cellulase after 2h incubation. The increased sensitivity of the mAb staining compared to silver staining also shows that there was some release of proteins from the sheath without enzyme (Fig. 3.1.2b, lane 5). This non-enzyme release was variable and never approached the level of enzyme-mediated release.

The cellulase preparation used in Fig. 3.1.2 clearly

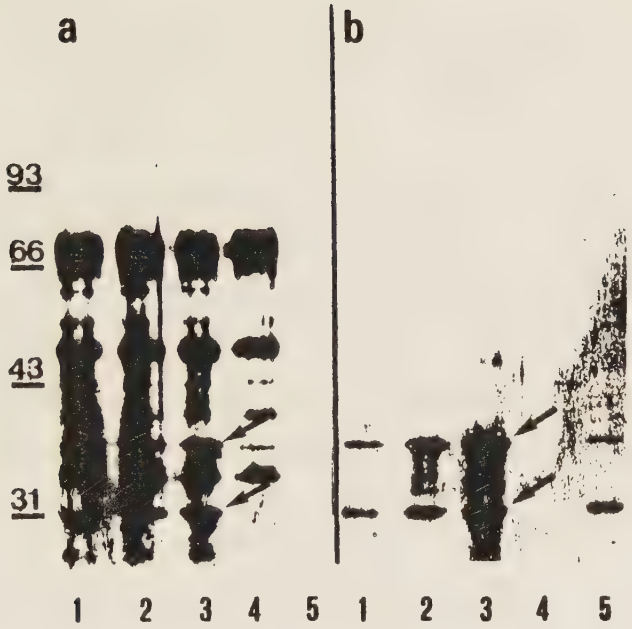


Figure 3.1.2: Cellulase release of sheath proteins. (a) Silver staining (b) MUD50 stained immunoblot of (a). Lanes: (1) sheath + cellulase (0.25 mg/ml), 0 time; (2) sheath + cellulase, 30 min.; (3) sheath + cellulase, 60 min.; (4) cellulase alone, 60 min.; (5) sheath alone, 60 min.. 10% acrylamide resolving gel.

contains many proteins, many of which may not be related to the cellulase activity. This raised the possibility that the release of proteins from the sheath by this preparation may have been due to an unknown, non-cellulase enzyme contaminant.

The possibility that contaminating protease may have been responsible was examined by pre-incubation of both the sheath and the enzyme preparation with the protease inhibitors pepstatin (25μg/ml) and PMSF (2mM) and their inclusion in the incubation buffer. No change in the pattern of protein release was seen (data not shown) although PMSF slightly inhibited cellulase activity and concurrently decreased the amount of protein released.

As an additional check, the crude cellulase preparation was partially purified by gel filtration chromatography on Sephacryl S-200. The sheath protein releasing activity and the cellulase enzyme activity co-eluted from the column (data not shown). This strongly implies that the release of sheath proteins by the crude enzyme preparation was due to cellulase activity alone. In view of this conclusion, the crude cellulase preparation was considered suitable for use in further experiments without prior purification and in the absence of protease inhibitors.

The apparent association of at least some sheath proteins with carbohydrate (ie cellulose) raised the possibility that they may be related to a family of endogenous D.discoideum lectins, the discoidins (see Bartles & Frazier, 1982). Purified discoidins (a gift from Prof. G. Gerisch) did not comigrate with the cellulase released proteins in SDS-PAGE

nor did they react with a range of mAb's which recognise the sheath proteins (data not shown).

3.1.3 Monoclonal Antibodies Identifying Sheath Proteins

The data presented in the preceding sections suggested the association of a number of proteins with the sheath. In order to probe the possible sheath specificity or sheath/slug cell sharing of these proteins, mAb's were raised against the sheath.

Initially, whole homogenised sheath was used as the immunogen. In these preliminary experiments, no hybrids positive for anti-sheath activity were obtained. Subsequently, mice were immunised with urea extracted sheath proteins. Urea rather than SDS extracted proteins were chosen because of the widely held view that antibodies raised in response to SDS denatured proteins will not recognise the native molecules.

Approximately 25 hybrids positive for anti-sheath activity (in the ELISA assay) were obtained from 4 fusions using urea-protein immunised mice. A small number of these hybrids were lost before they could be cloned and further characterised. All of the remaining hybrids secreted mAb's which showed one of the at least 3 classes of specificity shown in Fig. 3.1.3, with the majority showing the same pattern of protein recognition on immunoblots as MUD50. The

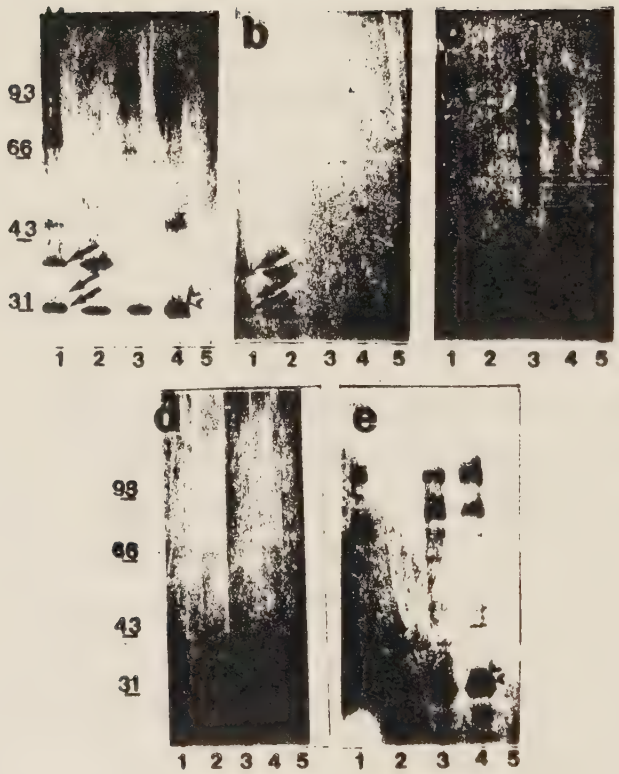


Figure 3.1.3: Immunoblots stained with antisheath mAb's. (a) MUD50; (b) MUD51; (c) MUD52; (d) MUD53; (e) MUD54. Lanes: (1) sheath; (2) cellulase released sheath proteins; (3) slug cells (4) slug cell crude plasma membranes; (5) vegetative amoebae. 10% acrylamide resolving gel.

other clear classes of mAb specificity are shown by MUD52 and MUD51/53. The specificity of MUD54 is uncertain, although it can be classified as MUD50-like. IgG subclasses (all of the mAb's are IgG) were not defined and no attempt was made to determine whether mAb's with the same specificity on immunoblots recognised the same antigenic determinant. No mAb which recognised a single protein was obtained.

All of the mAb's recognise sheath associated proteins of ca. 32kd, 34kd and 40kd in SDS and cellulase extracts of the sheath (arrows, Fig. 3.1.3, lanes 1). Proteins of these MW's were prominent in silver stained extracts of the sheath (Figs. 3.1.1). MUD51 and MUD 53 recognise only these proteins on immunoblots of sheath (Fig. 3.1.3b & d) but MUD50, MUD52 and MUD54 recognise a number of other sheath proteins (Fig. 3.1.3a, c & e). Comparison of staining of SDS and cellulase extracts by MUD50, MUD52 and MUD54 shows that not all sheath proteins are released by cellulase (Fig. 3.1.3a, c & e, lanes 1 & 2). Sheath proteins can, therefore, be divided into cellulase sensitive (released by cellulase) and cellulase resistant (not released by cellulase) classes.

Some (MUD50, MUD52 and MUD54), but not all, of the mAb's recognise slug cell proteins as well as sheath associated proteins (Fig. 3.1.3a, c & e, lanes 3 & 4) despite the use of sheath proteins as the immunising agent. Virtually all of the slug cell proteins recognised are membrane associated (Fig. 3.1.3, lane 4). Few of the slug cell proteins

Table 3.1.1 Summary of the mAb recognition of proteins on immunoblots. The numbers refer to the apparent MW (in kd) of each protein. Those marked with * may be common to sheath and slug cells.

mAb	Sheath	Slug Cells	Vegetative Cells
MUD50	108*, 85, 75-79, 50*, 40, 34, 32*, 26*	115, 107*, 95-100, 88, 73, 52, 50*, 45, 42, 32*, 26*	NO REACTION
MUD51	40, 34, 32	NO REACTION	NO REACTION
MUD52	124, 105, 97-100*, 78*, 60, 40, 34, 32	97-100*, 78-80*, 70, 46	NO REACTION
MUD53	40, 34, 32	NO REACTION	NO REACTION
MUD54	107*, 103*, 73-76, 60, 40, 34, 32*	108*, 103*, 79-82, 70, 43, 32*, 27	NO REACTION

recognised by the mAb's have the same apparent MW as mAb recognised sheath proteins (see Table 3.1.1). There is, therefore, extensive sharing of antigenic determinants between slug cells and the sheath but relatively little sharing of specific proteins.

One clear case of possible sharing of a specific protein as well as antigenic sites is the 32kd protein. A protein of this MW is heavily silver stained in both sheath and slug cell extracts (Fig. 3.1.1a). More importantly, a protein of this MW is strongly stained by MUD50 and MUD54 in both sheath and slug cells extracts (open arrows, Fig3.1.3a & e) but only in sheath by MUD51, MUD52 and MUD53. The basis of this partial mAb overlap of this protein is investigated in greater detail in succeeding chapters, as is the distribution of cellular proteins within the slug.

All of the antigenic determinants defined by the mAb's are specific to the developmental stage of D.discoideum's life cycle. No proteins were detected on immunoblots of vegetative cells (Fig. 3.1.3, lane 5).

3.1.4 Nature of the Antigenic Determinants Recognised

The nature of the antigenic determinants was examined by comparing the abilities of urea extracted sheath proteins and Pronase digests of them to inhibit the ELISA's of each mAb. The results are shown in Fig. 3.1.4. Pronase digestion completely destroyed the ability of sheath proteins to inhibit the ELISA's of MUD51 and MUD53, so these mAb's

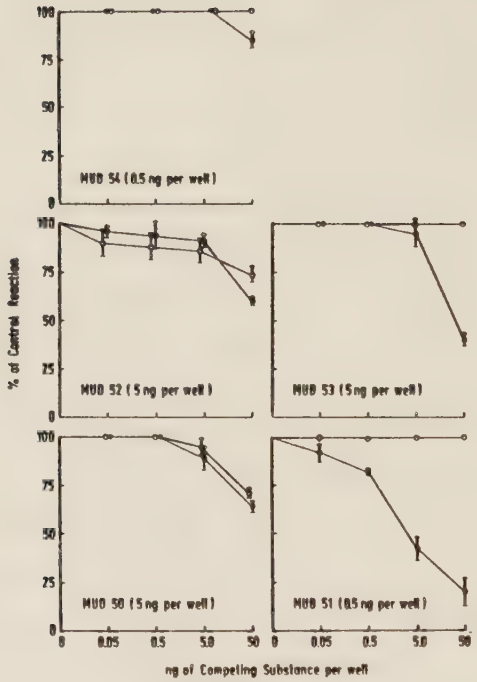


Figure 3.1.4: Competition ELISA. The control (no competing antigen) aborbance was taken as 100% and the absorbance of wells with competing antigens as a percentage of this value. For competing antigens, the amounts added refer to (a) the amount of protein for undigested material; or (b) the amount of protein before Pronase digestion for the glycopeptide fraction. Closed circles: sheath proteins as competing antigen; open circles; sheath glycopeptides as competing antigen. Bars represent standard error of the mean of 12 determinations.

recognise Pronase sensitive determinants. The ELISA's of MUD50 and MUD52 were equally inhibited by digested and undigested material and so recognise Pronase insensitive determinants. The ELISA of MUD54 was poorly inhibited by either fraction, although at higher concentrations of inhibiting material than shown this mAb also appears to recognise a Pronase insensitive determinant.

The uncertainty surrounding the real specificity of MUD54 (see 3.1.3) and the nature of the antigenic determinant which it recognises illustrate that not all mAb's are easily characterised. This mAb was not used extensively in this work for this reason.

That the inhibition of the ELISA's of MUD50 and MUD52 was due to the presence of low concentrations of undigested protein was considered to be unlikely because :

(a) Pronase digestion had no effect on the "specific activity" (the inhibitory activity of the Pronase digests per μg of protein before digestion) of the Pronase digested material in the ELISA

(b) MUD51 and MUD53 inhibition was completely abolished by Pronase digestion.

Two approaches were used to test this. Firstly, up to 100 times the amount of Pronase digested material necessary for 50% inhibition in the ELISA was immunoblotted. No staining was seen with any of the mAb's (data not shown).

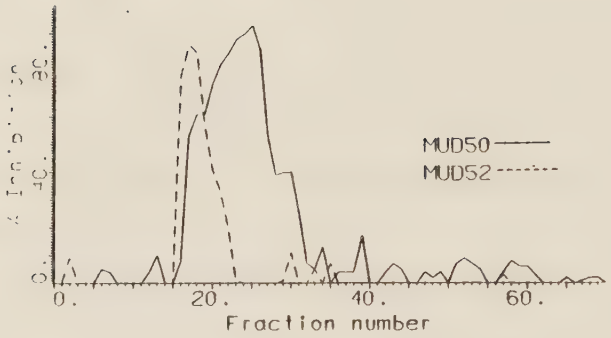


Figure 3.1.5: Sephadex G-50 chromatography of sheath glycopeptides. Each fraction was lyophilised then dissolved in the same volume as the initial sample applied to the column and tested in the competition ELISA of MUD50 and MUD51 (see Fig. 3.1.4). % inhibition was calculated as 100 - % control.

Secondly, the Pronase digested material was fractionated by Sephadex G-50 gel filtration (Spiro, 1972). No material able to inhibit the ELISA's of MUD51 or MUD53 was detected in any column fraction (data not shown). Material able to inhibit the ELISA of MUD50 was contained in a broad peak approximately one third of the way through the elution volume (the total volume available to solvent in the column) with a maximum of inhibitory activity in fraction 25 (Fig. 3.1.5). Material able to inhibit the ELISA of MUD52 was eluted as a sharper peak with a maximum in fraction 16 (Fig. 3.1.5). The two peaks overlap but have maxima in quite different positions. This, plus the differences in the proteins recognised by MUD50 and MUD52 (see Table 3.1.1), suggest that the two mAb's recognise different carbohydrate determinants. The proteins they recognise are, therefore, glycoproteins.

MUD51 and MUD53 recognise protein determinants.

3.1.5 Affinity Chromatography and Immunoprecipitation of Sheath Proteins

In order to determine whether the same 32kd, 34kd and 40kd proteins were recognised by each of the mAb's and to prepare material for peptide mapping studies, MUD50-recognised sheath proteins were separated from those not recognised by MUD50 by affinity chromatography on a MUD50-Sepharose column. The results of a typical column run are shown in Table 3.1.2.

Table 3.1.2: MUD50 Affinity Chromatography. [a]: 25ng of each fraction was added to the ELISA with 5ng of MUD50 or MUD52, or 0.5ng of MUD51, see Fig. 3.1.4).

Fraction	µg Protein	% Control ELISA[a]		
		MUD50	MUD51	MUD52
Starting material	240 (100%)	45	20	50
Unbound fraction	186.5 (76%)	100	100	75
Bound fraction	36.8 (15%)	60	20	60
Recovery	223.3 (91%)			

On the basis of recovery of protein from the column, ca. 15% of sheath proteins are recognised by MUD50 although this figure varied considerably between column runs (from ca. 10% to 80%). This may have been due to the low column loadings, which made assay of the protein content of the fractions difficult. Of more importance, however, is the clear observation that *no* proteins able to inhibit the ELISA of MUD50 remained in the unbound fraction ie. the column bound all MUD50 proteins.

For MUD52 the situation is different. Material in the MUD50-Sepharose unbound fraction is able to inhibit the ELISA of MUD52 ie. most, but not all, MUD52-recognised protein is bound by the column. This is in agreement with the data in Fig. 3.1.3, which showed that both mAb's stained the 32kd, 34kd and 40kd proteins but that some proteins were stained exclusively by one or the other.

In addition to binding all MUD50-recognised proteins, the MUD50-Sepharose column also bound all MUD51-recognised proteins, showing that all these proteins are also recognised by MUD50. This is in agreement with the data presented in Fig. 3.1.3, where both mAb's stained proteins of 32kd, 34kd and 40kd.

In order to determine whether all MUD50-recognised proteins were recognised by MUD51, a MUD51-Sepharose column was prepared and run under identical conditions to the MUD50-Sepharose column. Immunoblots of the bound and unbound

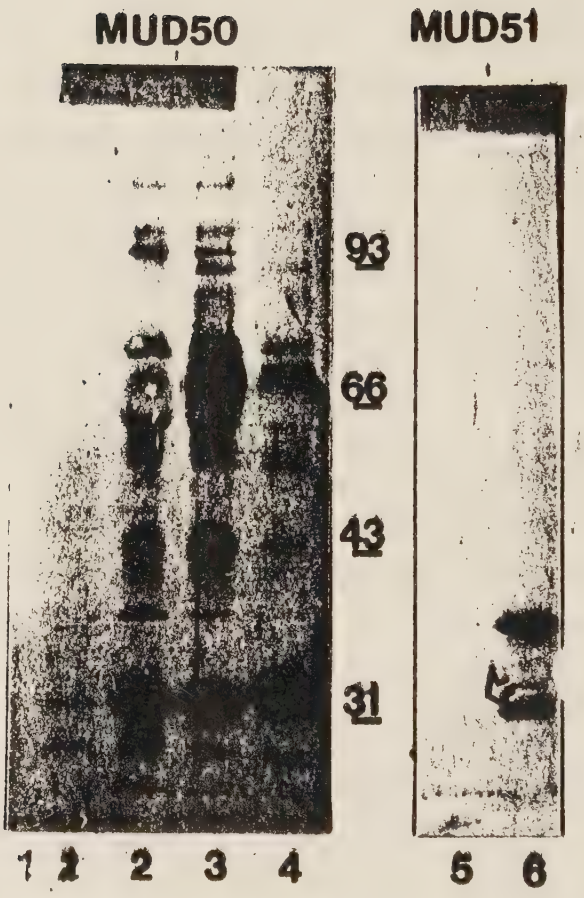


Figure 3.1.6: Immunoblots of mAb affinity column fractions. (a) MUD50 staining (b) MUD51 staining. Lanes; (1) MUD50-Sepharose, unbound; (2) MUD50-Sepharose, bound; (3) MUD51-Sepharose, unbound; (4) MUD51-Sepharose, bound; (5) MUD51-Sepharose, unbound; (6) MUD51-Sepharose, bound. All samples concentrated by acetone precipitation. 10% acrylamide resolving gel.

fractions from both the mAb-Sepharose columns are shown in Fig. 3.1.6. As expected from the ELISA data (Table 3.1.2) immunoblots of the MUD50-Sepharose *unbound* fraction do not stain with MUD50 (Fig. 3.1.6, lane 1). Similarly, no MUD51 staining is seen in the *unbound* fraction from the MUD51-Sepharose column (Fig. 3.1.6, lane 5) ie. *both* columns bind all proteins they recognise.

A large number of proteins were stained by MUD50 in the MUD51-Sepharose unbound fraction, but this is expected since MUD50 clearly recognised more proteins than MUD51 (see Fig. 3.1.3). However, a 32kd protein is stained by MUD50 in the *unbound* fraction from the MUD51-Sepharose column (solid arrow, Fig. 3.1.6, lane 3) despite the clear absence of a MUD51 stained protein in the same fraction (open arrow, Fig. 3.1.6, lane 5). Thus, not all MUD50-recognised 32kd protein is recognised by MUD51. There is clearly a MUD50, 32kd protein which is not recognised by MUD51 and is not bound by the MUD51-Sepharose column. The identity of this protein is further investigated in the Chapter 3.2.

When the bound fractions from the columns were examined the expected pattern of MUD50 staining of the MUD51-Sepharose bound proteins was seen (Fig.3.1.6, lane 4; the doublet at 32kd is an artifact that was occasionally seen in acetone precipitated affinity purified material and the weak staining in the 65-70kd region due to slight carry-over of samples during loading).

To check the affinity chromatography results concerning the cross-reactivities of MUD50 and MUD51, and to extend these results to include MUD52, sheath proteins were

immunoprecipitated using SAC (Kessler, 1975).

No non-specific binding of sheath proteins to SAC in the absence of mAb occurred (Fig. 3.1.7, lane 2), although considerable background staining due to SAC itself was observed in some experiments when MUD50 was used as the precipitating mAb (Fig. 3.1.7a). This could be decreased but not consistently eliminated by pre-treatment of the SAC with SDS and β-mercaptoethanol (Richert et al., 1979).

When sheath proteins were immunoprecipitated with MUD50, MUD51 or MUD52 and the immunoblot stained with MUD50 (Fig. 3.1.7a), the 32kd and 40kd proteins (arrows) were stained in each case. This is in agreement with the affinity chromatography data (see above) and also demonstrates that the proteins precipitated with MUD52 are recognised by MUD50. (The precipitated proteins are equivalent to the *bound* fraction of an affinity column). Staining of the same material with MUD51 (Fig. 3.1.7b) confirms the cross-reactivity of these mAb's with the 32kd and 40kd proteins. The affinity chromatography and SAC precipitation data are summarised in Table 3.1.3.

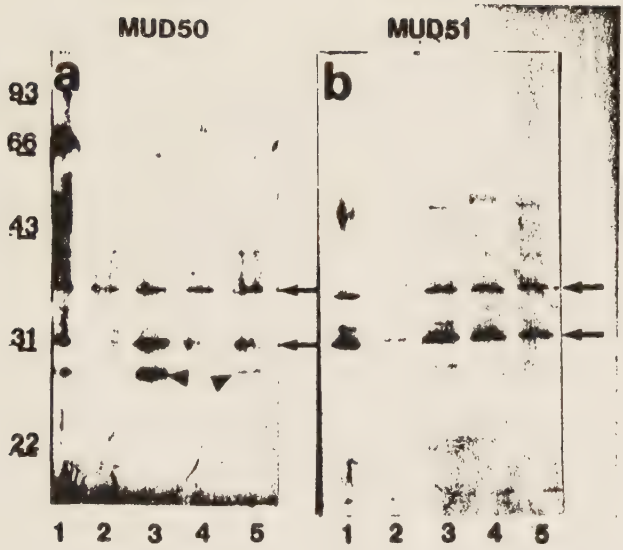


Figure 3.1.7: Immunoblots of SAC-precipitated sheath proteins. (a) MUD50 staining (b) MUD51 staining. Lanes; (1) sheath proteins before immunoprecipitation; (2) no antibody control ie. sheath + SAC but no mAb; (3) MUD50 SAC immunoprecipitate; (4) MUD51 SAC immunoprecipitate; (5) MUD52 SAC immunoprecipitate. 12% acrylamide resolving gel.

The solid triangular symbols indicate the position of the IgG light chains (see Fig. 3.2.5) and are not sheath proteins.

Table 3.1.3: Summary of affinity chromatography and SAC immunoprecipitation data. [a] Fractions from the affinity columns; un: unbound, bo: bound. [b] data for the MUD51 staining of MUD50-Sepharose column fractions was not shown in Fig. 3.1.6. neg.: no staining detected.

STAINING	COLUMN FRACTION[a]			SAC PRECIPITATING mAb		
mAb	MUD50un	MUD50bo	MUD51un	MUD50	MUD51	MUD52
MUD50	neg.	32kd	32kd	32kd	32kd	32kd
		40kd	neg.	40kd	40kd	40kd
MUD51	neg.[b]	32kd	neg.	32kd	32kd	32kd
				40kd	40kd	40kd

3.1.6 HPLC Peptide Mapping of the 32kd and 40kd Sheath Proteins

The recognition of the 32kd, 34kd and 40kd proteins by all anti-sheath mAb's suggested that they may be related at the polypeptide sequence level as well as at the antigenic level. Bound material from the MUD50-Sepharose column was

separated by SDS-PAGE and stained with Coomassie Blue. The 32kd, 34kd and 40kd proteins were cut from the gel and radioiodinated. To check for degradation or loss of antigenicity during the lengthy preparative procedure a small portion of each [^{125}I]-protein was re-electrophoresed, transferred to nitrocellulose then autoradiographed and immunoblotted. No changes in MW or recognition by mAb were seen (data not shown).

Following radioiodination, the gel slices were digested with trypsin. The efficiency of release of radioactivity from the slices (tryptic peptides) is shown in Table 3.1.4. The release was slightly less than that reported by Elder et al. (1977).

HPLC peptide maps of the tryptic peptides of the 32kd and 40kd proteins are shown in Fig. 3.1.8. Comparison of the peptide maps of the 32kd and 40kd proteins shows that they have few peaks (peptides) in common (Fig. 3.1.8a). For both proteins, nearly all of the major peaks (solid arrows) are specific for the protein concerned, although in some cases there are minor peaks from one protein under major peaks from the other (eg. there is a small peak from the 40kd protein peak under the major peak from the 32kd protein at R_t= 51 min.). The three late 40kd protein peaks are particularly characteristic, since there are no 32kd protein peaks in this region of the chromatogram (Fig. 3.1.8a, solid arrows).

Table 3.1.4: Release of tryptic peptides from gel slices. The 34kd sheath protein labelled poorly with the Bolton-Hunter reagent and was not included in the analysis.

Protein	Before Trypsin (c.p.m.)	c.p.m. released	%
Ovalbumin	933,985	524,535	56
Sheath 40kd	734,398	294,576	40
Sheath 32kd	875,548	367,797	42

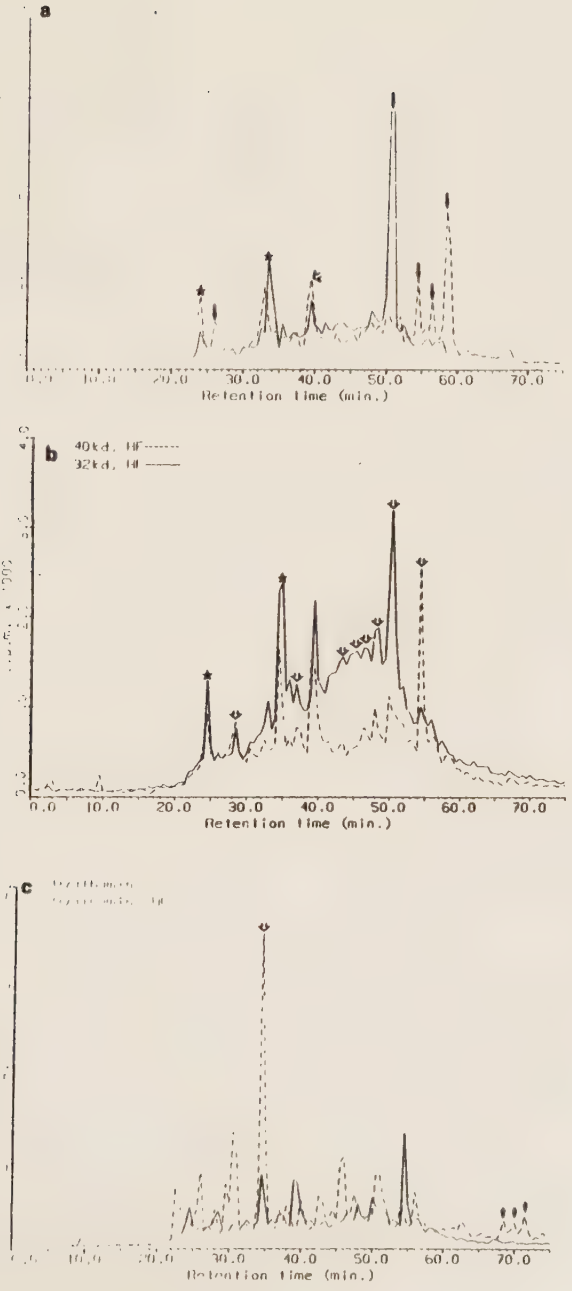


Figure 3.1.8: HPLC peptide mapping of sheath proteins.

The only unambiguous case of overlapping major peaks is at R_t= 39 min. (open arrow, Fig. 3.1.8a). The apparently common peaks with R_t's of 24 min. and 33 min. (marked with stars) are not due to peptides but rather to radioiodination of a component of the polyacrylamide gel slices (they are present in the chromatograms of gel slices which contained no protein, data not shown; see Kelly et al., 1981).

The 32kd and 40kd proteins are glycoproteins (see 3.1.4), so it was possble that the differences seen in their peptide maps may have been partly due to differences in glycosylation. Consequently, the peptides from both proteins were deglycosylated using HF (Mort & Lamport, 1977) and the HPLC maps of the deglycosylated peptides compared (Fig. 3.1.8b).

Following HF treatment a number of striking changes occurred, resulting in peptide maps for the two proteins which no longer differed significantly in the position of most peaks, although there were still differences in peak heights. For example, the three late 40kd protein specific peaks (solid arrows, Fig. 3.1.8a) disappeared and were replaced by a single late deglycosylated peak (open arrow at R_t= 55 min., Fig. 3.1.8b) with the same R_t as a new 32kd protein peak (R_t= 55 min.). The major 32kd protein peak remained at R_t= 51 min., but was joined at this position by a prominent 40kd peak (open arrow at R_t= 51 min., Fig. 3.1.8b). In addition, a series of new common peaks precedes the major R_t= 51 min. peak and a new common peak also appears between the starred artifact peaks (all new common peaks in 3.1.8b are marked with open arrows). There was also an apparent increase in the baseline of the 32kd protein

peptide map following treatment with HF which makes interpretation of the differences in absolute peak heights between the 32kd and 40kd deglycosylated peptide maps difficult.

As a control of the effect of HF on peptide maps, ovalbumin peptides were also compared before and after treatment (ovalbumin is a glycoprotein). As was the case for the sheath proteins, deglycosylation caused major changes in the peptide map of ovalbumin (Fig. 3.1.8c). Whether all of the changes seen following treatment with HF are due solely to deglycosylation was not investigated, although under the conditions used there should not have been any effect on non-sugar components of the glycoproteins (see Mort & Lamport, 1978).

CHAPTER 3.2: SHEATH PROTEIN POLYMORPHISMS

3.2.1 Polymorphisms of MUD50-Recognised Proteins

Given the availability of a large number of wild isolates of D.discoideum and the specific probes provided by the mAb's, a screen of the sheath and slug cell proteins of a number of wild isolates was made using MUD50 in the hope of detecting protein polymorphisms. The aim was to try to obtain a way of analysing the sheath proteins genetically.

Of the 21 wild isolates screened, 6 differed detectably from the wild type (defined as the most common form of MUD50 reactivity, see Table 3.2.1). Immunoblots of a selection of the 21 wild isolates (showing 5 of the 6 polymorphisms) are shown in Fig. 3.2.1.

Three types of polymorphism were observed in the immunoblots of slug cells from the wild isolates (Fig. 3.2.1a, a fourth is shown in Fig. 3.2.1i). The two most frequent (4 out of 6) were upward or downwards shift of ca. 2kd in the MW of the 32kd slug cell protein (upward: HU188 & WS578; downward: Dd61 & WS380B; Fig. 3.2.1a, lanes 3, 5, 1 & 8 respectively). In addition to the 32kd protein, two smaller proteins of ca. 27kd and 22kd underwent similar MW shifts in all 4 cases (arrows, Fig. 3.2.1). These proteins stain very faintly and are not always visible. They may represent either precursors or degradation products of the 32 kd protein. The observation that the difference in MW from the wild type is the same for all proteins involved in

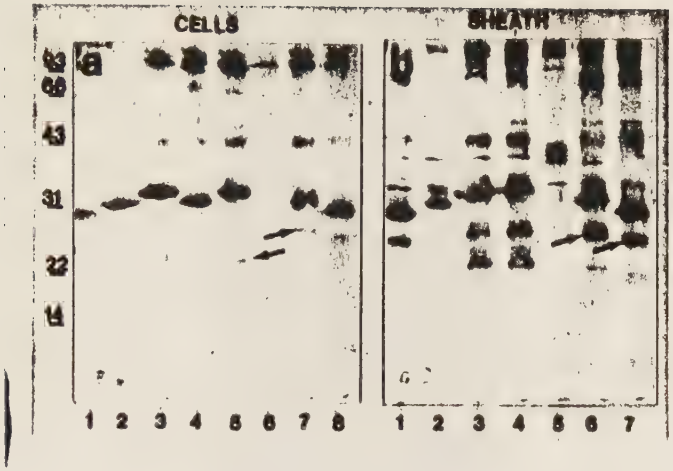


Figure 3.2.1: Screening for MUD50 polymorphisms in wild isolates. (a) Slug cells. Lanes: (1) Dd61; (2) HU182; (3) HU188; (4) HU1628 (not a wild isolate, see 3.2.5); (5) WS576; (6) WS584; (7) WS269A; (8) WS380B. (b) Sheath. Lanes: (1) Dd61; (2) HU182; (3) HU188; (4) WS576; (5) WS584; (6) WS269A; (7) WS380B. 15% acrylamide resolving gel.

all 4 polymorphic isolates is striking.

When the sheath of the same wild isolates was examined the same pattern of 32kd protein polymorphism emerged (Fig. 3.2.1b, lanes 1, 3, 4 & 7). The 32kd protein of Dd61 was decreased to 30kd in Dd61 and WS380B and increased to 34kd in HU188 and WS576. As in slug cells, coordinate changes in the MW of two smaller proteins were seen in the sheath.

The third type of polymorphism shown in Fig. 3.2.1 is exhibited by WS584. The slug cells of this isolate do not contain any proteins recognised by MUD50 (Fig. 3.2.1a, lane 6) and its sheath has far fewer MUD50-recognised proteins than the wild type (Fig. 3.2.1b, lane 5). In addition, those proteins which are present are of different MW to the wild type sheath proteins.

The fourth type of polymorphism of a sheath protein was found in WS472 (see section 3.2.7, Fig. 3.2.11).

A rapid, relatively crude method was used to prepare the sheath shown in Fig. 3.2.1b so that unequal loading of the lanes occurred and contamination of the sheath with slug cells was possible. This was especially relevant for the 32kd protein polymorphism, since it involves a protein which may be common to sheath and slug cells (see 3.1.3). To check this more thoroughly, sheath from the wild type and Dd61 (30kd) and WS576 (34kd) was prepared in the standard way (see 2.3) and immunoblotted. Comparison of the MUD50 recognised sheath proteins in this case shows:

Figure 3.2.2: MUD50 stained sheath from polymorphic wild isolates. Lanes: (1) Dd61; (2) NP84 (the wild type, derived from the V12 wild isolate); (3) WS576. 12% acrylamide resolving gel.

(a) that the 30kd and 34kd forms of the protein are in the sheath (open arrows, Fig. 3.2.2) and

(b) that in addition to the polymorphic form of the protein, there is a normal 32kd form in the sheath (solid arrows, Fig. 3.2.2).

In the wild type (strain NP84) these two proteins presumably have the same MW and are not separated on one dimensional SDS gels (see 3.2.4). Comparison of Figs. 3.2.1a and 3.2.2 suggests that the "normal" 32kd form of the protein is not found in slug cells of Dd61 and WS576.

3.2.2 Polymorphic and Non-polymorphic 32kd Proteins

The existence of polymorphic and non-polymorphic 32kd MUD50-recognised proteins and the sheath specificity of one form was demonstrated more clearly with MUD1 and MUD51 staining of slug cell and sheath immunoblots.

MUD51, a sheath specific mAb, has been described (3.1.3). MUD1 was obtained from a fusion using spleen cells from a mouse which had been immunised with slug cells rather than sheath (Gregg et al., 1982) and specifically recognises prespore cells in the slug (Gregg et al., 1982; Krefft et al., 1983a & b). This mAb recognises a single protein of 32kd in slug cell extracts (Krefft et al., 1983a & b).

In order to facilitate their description the various forms of the 32kd protein(s) and the loci coding for them have been named in accordance with the Demerec system used for bacterial genetics (Demerec et al., 1966). This nomenclature is described in Table 3.2.1. The data

supporting the table are presented in the following sections.

Table 3.2.1: Nomenclenture and mAb reactivity of polymorphic and non-polymorphic proteins. The locus *psp*A codes for the polymorphic protein which will be referred to as PsA. The locus *shp*A codes for the non-polymorphic protein which will be referred to as ShA. Note that there are 3 different sized products of the *psp*A locus. It has been assumed that there is only one *shp* locus but this could not be demonstrated genetically.

LOCUS/ALLELE	MW	WILD ISOLATE	PROTEIN	mAb
*psp*A350	32kd	Wild type	PsA	1, 50
*psp*A351	30kd	Dd81, WS380B	PsA	1, 50
*psp*A352	34kd	WS576, HU188	PsA	1, 50
*shp*A350	32kd	All except WS584	ShA	50, 51, 52

When the same slug cell extracts that were used in Fig. 3.2.1a were immunoblotted and stained with MUD1, essentially the same pattern of PsA polymorphism as that seen with MUD50

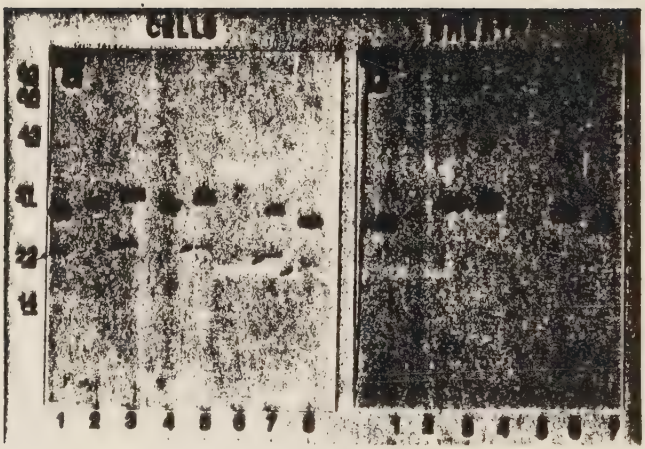


Figure 3.2.3: MUD1 stained immunoblots of wild isolates. (a) Slug cells. (b) Sheath. Lanes: as in Fig. 3.2.1. 15% acrylamide resolving gel.

was observed: Dd61 and WS380B have a 30kd PsA protein and HU188 and WS578 have a 34kd PsA protein (Fig. 3.2.3a, lanes 1, 8, 3 & 5 respectively). Also as in Fig. 3.2.1a, there were lower MW proteins which showed the same changes (small arrows, Fig. 3.2.3a) as PsA. Included in this figure is the slug cell extract of WS584. No WS584 slug cell proteins stained with MUD1 (Fig. 3.2.3a, lane 6).

MUD1 staining of sheath extracts showed the same pattern of PsA polymorphism as MUD1 staining of slug cells and the same PsA pattern as revealed by MUD50 staining; ie there is an exact parallel between MUD1 and MUD50 staining of PsA in sheath and slug cells. This demonstrates that:

(a) MUD1 and MUD50 recognise the same polymorphic PsA protein and

(b) the PsA protein is present in both slug cells and the sheath. The sheath/slug cell antigenic overlap noted for the 32kd protein in 3.1.3 is due, therefore, to the presence of PsA in the sheath and slug cells.

Data was also presented in the preceding chapter (section 3.1.5) that there was a MUD50-recognised 32kd protein which was not bound by the MUD51-Sepharose column. MUD51 staining of the polymorphic isolates' sheath clearly shows that the PsA protein is not stained by this mAb (Fig. 3.2.4a, the arrows indicate the MW's of the polymorphic forms of PsA). The non-binding 32kd protein referred to above may, therefore, be PsA. Staining of the same material with MUD52

Figure 3.2.4: Immunoblots of wild isolate sheath stained with (a) MUD51 and (b) MUD52. Lanes: (1) Dd61; (2) NP84; (3) WS576. The high background staining is due to "exchange" of mAb during the washing steps of the staining procedure.

shows that, like MUD51, it does not recognise PsA. The protein recognised by these two antibodies is the non-polymorphic 32kd protein seen in Fig. 3.2.2 ie. ShA. These data are summarised in Table 3.2.2.

Table 3.2.2: Summary of mAb reactivity and location of PsA and ShA. Note that PsA is of the same MW in sheath and cells and that ShA is non-polymorphic with a MW of 32kd. The + and - symbols refer to recognition or non-recognition, respectively, by the mAb in question.

	MUD1		MUD50		MUD51/52	
	PsA	ShA	PsA	ShA	PsA	ShA
NP84						
Slug cells	+(32kd)	-	+	-	-	-
Sheath	+(32kd)	-	+	+	-	+
Dd61						
Slug cells	+(30kd)	-	+	-	-	-
Sheath	+(30kd)	-	+	+	-	+
WS576						
Slug cells	+(34kd)	-	+	-	-	-
Sheath	+(34kd)	-	+	+	-	+

Slug cells and sheath from the wild isolates summarised in Table 3.2.2. were also immunoblotted and stained with MUD53 and MUD54. The same pattern of reactivity as MUD50 was seen with MUD54 while MUD53 was the same as MUD51.

ConA-peroxidase staining of sheath proteins from these wild isolates showed no differences to the wild type (data not shown).

3.2.3 SAC Immunoprecipitation of PsA and ShA

The existence of polymorphic (PsA) and non-polymorphic (ShA) proteins in the sheath can be confirmed by taking advantage of their different mAb reactivity to allow their selective immunoprecipitation. These experiments could only be performed with WS576 (which has the high MW form of PsA). The presence of staining due to the IgG light chains in the region of the gel where the 30kd PsA would be found obscured the result with Dd61 (see Figs. 3.1.7 and 3.2.5).

MUD50 staining of immunoblotted SAC immunoprecipitated sheath proteins clearly shows that MUD50 precipitates the PsA protein (solid arrow, Fig. 3.2.5a, lane 2) but MUD51 does not (Fig. 3.2.5a, lane 3). Staining of the same material with MUD51 also shows that PsA is not recognised by this mAb (Fig.3.2.5b, lane 2, the solid arrow indicates the expected position of PsA). The open arrows in this figure indicate the IgG heavy and light chains (SAC binds IgG so that in lanes 4 & 5, which contain no sheath proteins, only the IgG chains are stained).

3.2.4 Two Dimensional Electrophoresis of Sheath Proteins

The existence of PsA and ShA proteins in the polymorphic

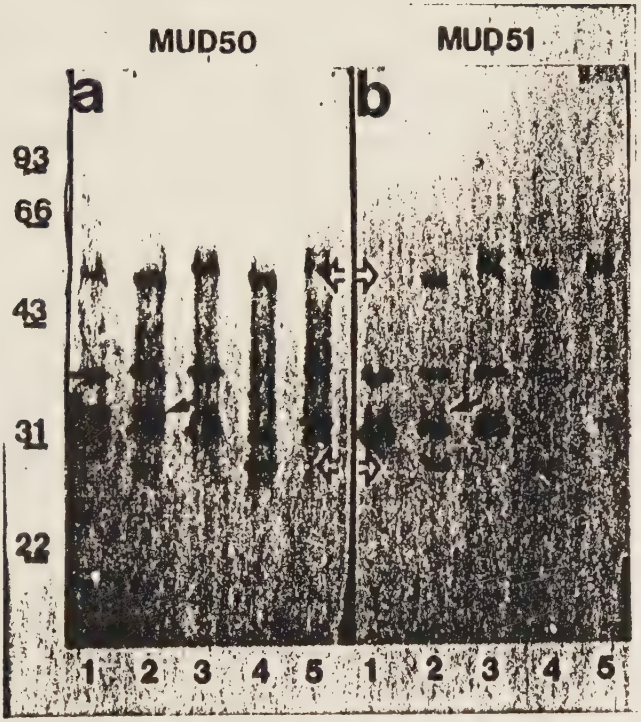


Figure 3.2.5: Immunoblots of SAC-precipitated sheath proteins stained with (a) MUD50 and (b) MUD51. Lanes: (1) sheath proteins before SAC-precipitation; (2) MUD50 SAC-precipitates; (3) MUD51 SAC-precipitates; (4) MUD50 no antigen control ie. MUD50 + SAC without sheath proteins; (5) MUD51 no antigen control. The open arrows indicate the IgG light and heavy chains.

wild isolates implies that there should also be PsA and ShA proteins in the sheath of the wild type. The 32kd protein which is recognised by MUD50 but not MUD51 may be PsA (see above) but in order to demonstrate this directly PsA must be unambiguously identified in the wild type. Two dimensional electrophoresis is a more sensitive means of separating proteins than one dimensional SDS-PAGE (O'Farrell, 1975) since separation is based on isoelectric point (pI) as well as MW. It was hoped that this technique would allow identification of the wild type PsA protein. Immunoblots of 2-D gels of sheath proteins are shown in Fig. 3.2.6.

The MUD50 stained immunoblots of sheath proteins are surprisingly complex (Fig. 3.2.6a, c & e).The majority of the bands stained on one dimensional gels (extreme right) are resolved into multiple spots. Of particular importance is that the wild type (strain NP84) 32kd protein is resolved into at least three distinct spots in the range pH 4.5-5.0 (arrows, Fig. 3.2.6c).

Comparison of MUD50 and MUD51 staining of NP84 sheath proteins shows that the two most basic 32kd spots are not stained by MUD51 (dashed circles, open arrows, Fig. 3.2.6d). In Dd61 and WS576 it is the two most basic spots which are polymorphic for MW (Fig. 3.2.6a & e) and, as expected, neither of these spots stain with MUD51 (Fig. 3.2.6b & f). It is clear, therefore, that the wild type possesses protein(s) homologous to PsA which behave identically in terms of mAb reactivity and pI on 2-D gels to the PsA

Figure 3.2.6: Two dimensional gel analysis of sheath proteins; immunoblots stained with (a), (c) and (e) MUD50 and (b), (d) and (f) MUD51. Gels (a) and (b) are Dd61, gels (c) and (d) are NP84 and gels (e) and (f) are WS576. The pH range in the first dimension was pH 4 -8. The second dimension was 12% acrylamide in the resolving gel. A one dimensional gel was run in parallel on the extreme right of each panel.

defined in the polymorphic wild isolates.

The PsA and ShA proteins show a number of similarities in the isoelectric focussing dimension of the gels. In the wild type and Dd61 the most acidic ShA spot has the same pI as the most basic PsA protein and in all cases the spacing between the spots is similar. A more striking similarity is that the polymorphism does not have a detectable influence on the pI's of the PsA proteins despite their difference in MW. The addition/deletion which led to the polymorphism must, therefore, be neutral. The reason for the resolution of PsA and ShA into multiple spots is unknown. A general comparison of the MUD50 stained 2-D gels shows that there are few differences between the different wild isolates other than the PsA polymorphism MW differences. Therefore, the addition/deletion must also be specific for the PsA protein.

3.2.5 Mapping of the *psp*A Locus

The polymorphic Dd61 *psp*A351 locus (which is responsible for the 30kd form of PsA) was assigned to a linkage group using standard parasexual genetic techniques (Newell, 1982; the genetic work was done in collaboration with Dr. D. Welker). A Dd61 x HU1628 diploid was constructed by fusing Dd61 with HU1628, a haploid strain derived from the HC4 wild isolate which carries markers on 6 of the 7 linkage groups (Welker & Williams, personal communication). Diploid cells were selected from haploids on the basis of dominant cobalt resistance and ability to grow on B.subtilis rather than K.aerogenes, and their ploidy checked on the basis of spore size (diploid spores are approximately twice as large as

haploid spores, Stenhouse & Williams, 1981).

When such diploids are grown in the presence of an appropriate concentration of an anti-microtubule agent (eg. the anti-fungal agent Benlate, Williams & Barrand, 1978; or thiabendazole, an anti-helminthic agent, Welker & Williams 1980) they rapidly revert to the haploid state by random loss of chromosomes. The resultant haploid segregants contain, therefore, a random mixture of chromosomes drawn from the diploid. Aneuploids, while transiently present, are not sufficiently stable to form colonies (Brody & Williams, 1974). The random loss of chromosomes during haploidisation means that a given haploid segregant may contain any random combination of the linkage groups of the parental haploids. Consequently, if two phenotypes are always associated in a range of segregant haploids, the loci responsible for the phenotypes must be on the same linkage group. Since this is done in a parasexual rather than a sexual system, the incidence of spontaneous crossing over is rare (10^{-3}–10^{-4}, see also below) and does not interfere with the assignment of loci to linkage groups.

Slug cells of a selection of haploid segregants from the Dd61 x HU1628 diploid were immunoblotted and stained with MUD1 (Fig. 3.2.7a). In all haploids, only one form of PsA was seen; either the 30kd (HU2245 and HU2259) or the 32kd form (HU2250, HU2236, HU2237 and HU2267) but never both. This is particularly important for HU2245, which contains only one linkage group from Dd61, and for HU2250, which contains only one HU1628 (wild type) linkage group. The simplest explanation for this pattern of segregation is that

the 30kd PsA and 32kd PsA proteins are the products of different alleles at a single locus.

Examination of the genotypes of the various segregants (Table 3.2.3) shows that all those with the 30kd PsA are sensitive to cycloheximide (ie *cyc*A+) and all those with the 32kd PsA are cycloheximide resistant (*cyc*A-). The cycloheximide resistance locus (Katz & Sussman, 1972) is on linkage group I (Williams et al., 1974). Consequently, the *psp*A locus must also be on linkage group I.

Sheath from the same segregants was also immunoblotted and stained with MUD1 (Fig. 3.2.7b). In this case HU1628 was replaced by its "parent" NC4 because it contains mutation(s) which lead to premature fruiting and hence does not form migratory slugs. The segregants HU2245 and HU2237 were omitted for the same reason. The pattern of PsA segregation in the sheath of the strains which could be examined was the same as in slug cells ie. the same locus is responsible for the PsA polymorphism in the sheath as in slug cells.

Table 3.2.3: Genotypes of parental and haploid segregants in the Dd61 x HU1628 cross. The diploid DU2885 is heterozygous at all loci, including *psp*A (see 3.2.6). All loci are recessive (except the dominant cobalt resistance, which is probably associated with a plasmid, Metz et al., 1983).

STRAIN	I	II	III	IV	VI	VII	?
	cycA	acrA	bsgA	whiC	manA	tsgK	pspA
Dd61	+	+	+	+	+	+	-
HU1628	-	-	-	-	-	-	+
DU2885	+/-	+/-	+/-	+/-	+/-	+/-	+/-
HU2245	+	-	-	-	-	-	-
HU2250	-	+	+	+	+	+	+
HU2238	+	+	+	-	+	+	+
HU2237	-	+	-	+	-	-	+
HU2259	+	+	-	+	-	-	-
HU2267	-	+	+	-	-	-	-

Figure 3.2.7: MUD1 stained immunoblots of (a) slug cells and (b) sheath from the haploid segregants shown in Table 3.2.3. Lanes: (1) HU1628 (parent); (2) Dd61 (parent); (3) HU2250; (4) HU2245; (5) HU2236; (6) HU2237; (7) HU2259; (8) HU2267. Note that in (b) HU2245 and HU2237 are omitted because they do not form migratory slugs (see text). 12% acrylamide resolving gel.

To confirm that the assignment of *psp*A based on MUD1 staining of immunoblots also applied for MUD50, and that ShA was not involved, slug cells and sheath from the parents and two haploid segregants were immunoblotted and stained with MUD1, MUD50 and MUD51. The segregants HU2245 and HU2250 were chosen because they carry linkage group I from one parent with all other linkage groups from the other parent (see Table 3.2.3). For the reasons outlined above HU1628 and HU2245 were replaced by NC4 and HU2259, respectively, for the sheath immunoblots.

In both cells and the sheath, the MUD1 and MUD50 staining are in agreement (Fig. 3.2.8a & b, d & e). Both show HU2250 as having the wild type 32kd PsA (Fig. 3.2.8a, b, d & e, lanes 1 & 3) and HU2245 (HU2259) as having the polymorphic 30kd PsA (solid arrows, Fig. 3.2.8a, b, d & e, lanes 2 & 4) in both slug cells and sheath. The smaller occasionally stained proteins which undergo coordinated MW changes in the polymorphic wild isolates are relatively clearly seen in the MUD50 immunoblot (open arrows, Fig. 3.2.8b). The observation that these proteins follow the same genetic segregation as PsA supports the proposal that they are either precursors or degradation products of PsA.

As expected, MUD51 did not stain slug cell immunoblots (Fig. 3.2.8c) and stained only the non-polymorphic ShA protein in the sheath (Fig3.2.8f). The light staining in of the HU1628 slug cell immunoblot (Fig3.2.8c, lane 1) was almost certainly due to the presence of some late aggregates which contain stalk cells in the "slug" cultures this extract was made from (see Chapt. 3.3). Comparison between

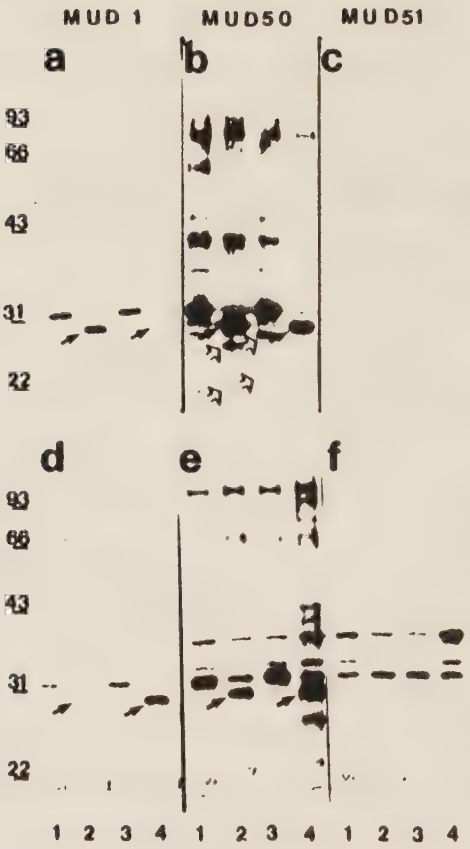


Figure 3.2.8: Immunoblots of parental and segregant haploids stained with (a) and (d)MUD1, (b) and (e) MUD50 and (c) and (f) MUD51. Panels (a - c) are slug cells and (d - f) are sheath. Lanes in (a - c): (1) HU1628; (2) Dd61; (3) HU2250; (4) HI2245. In (d - f), HU1628 is replaced by NC4 and HU2245 by HU2259 (see text). 12% acrylamide resolving gel.

MUD51 and MUD50 staining clearly demonstrates the MUD50 staining of the ShA protein in the polymorphic strains (Fig. 3.2.8e & f).

3.2.6 Mapping with Recombinant Diploids

The assignment of *pspA* to linkage group I using segregation of a diploid can be confirmed by mapping the same locus using mitotic crossing over. The majority of genetic markers are recessive and are not expressed when present in the heterozygous condition (eg. DU2885 is *cyoA+/cyoA-* and cycloheximide sensitive, see Table 3.2.3). By applying the correct selective pressure (eg. selection for cycloheximide resistance) diploids which have become homozygous for cycloheximide resistance via mitotic crossover are selected, generally at a frequency of 10^{-3}-10^{-4}. All other loci distal to the crossover will also become homozygous. This is shown schematically in Fig. 3.2.9.

This method requires that the linkage group of the locus in question be known so that the correct selective conditions are chosen, but it is much faster than the usual method of segregating a diploid. The latter requires the cloning and establishment of genotypes for a large number of haploid segregants (Newell, 1982). Since the linkage of *pspA* was known from the work described in the preceding section using the *pspA351* allele, diploids were constructed with HU1628 for the other *pspA* polymorphic wild isolates and selected for recombinant diploids on cycloheximide. Cycloheximide resistant recombinant diploids were obtained in all cases (see Table 3.2.4).

Figure 3.2.9: Schematic representation of mitotic crossing over leading to cycloheximide resistant (cyc-), 32kd PsA (psp+) recombinant diploids.

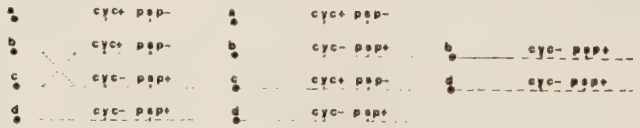


Table 3.2.4: Haploid parents, diploids and cycloheximide resistant recombinant diploids.

HAPLOID PARENTS	DIPLOID	RECOMBINANT
HU1628 x Dd61	DU2885	DU2914
HU1628 x HU188	DU2873	DU2913
HU1628 x WS380B	DU2889	DU2915
HU1628 x WS576	DU2900	DU2916

All of the diploids are heterozygous at *psp*A and express both forms of PsA (Fig. 3.2.10a & b, lanes 2, 4, 6 & 8). For example, DU2873 expresses the wild type PsA (32kd) and the polymorphic PsA (34kd, Fig. 3.2.10a & b, lane 2). In contrast, the cycloheximide resistant recombinant diploids express only the wild type (HU1628) PsA protein, regardless of the identity of the other haploid parent (Fig. 3.2.10a & b, lanes 1, 3, 5 & 7; the expected position of the polymorphic protein in the recombinant diploids is indicated by the solid arrows). Thus the mitotic crossover which resulted in cycloheximide resistance (the HU1628 phenotype) simultaneously resulted in homozygosity at *psp*A. This means that both loci must be on the same linkage group ie. linkage group I, and that all the *psp*A polymorphisms map to the same linkage group (and probably the same locus).

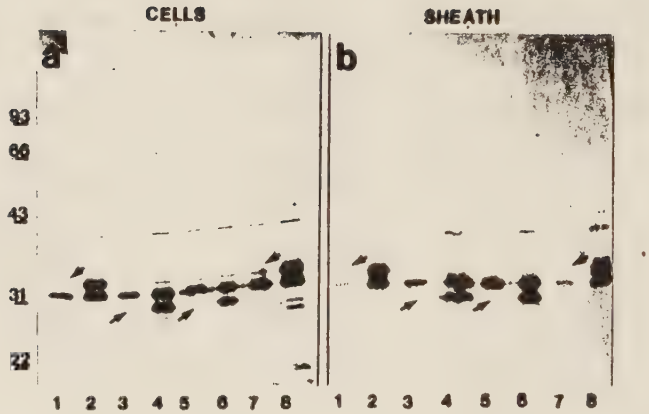


Figure 3.2.10: MUD1 stained immunoblots of recombinant and parental diploid (a) slug cells and (b) sheath. Lanes: (1) DU2913; (2) DU2873; (3) DU2914; (4) DU2885; (5) DU2915; (6) DU2889; (7) DU2916; (8) DU2900. 12% acrylamide resolving gel.

In order to obtain cycloheximide resistant recombinants, the crossover must occur between *cyoA* and the centromere (see Fig. 3.2.9). If *psoA* was located proximal to *cyoA* (closer to the centromere) then crossing-over between the two loci could be expected. Such crossovers would result in cycloheximide resistant recombinants which remained heterozygous for *psoA*. No such diploids were found, suggesting that the order shown in Fig. 3.2.9 may be correct or that the two loci are close to each other.

As has been seen in all comparisons of sheath and slug cell PsA, no differences between sheath and slug cells was detected in any of the recombinant diploids (Fig. 3.2.10a & b).

3.2.7 The WS472 and WS584 Polymorphisms

In addition to the PsA polymorphism analysed in the preceding sections, two further sheath protein polymorphisms were found. The WS584 polymorphism results in the loss of PsA and all slug cell MUD50 antigenic determinants (see 3.2.1 and 3.2.2). A more detailed analysis of this polymorphism is shown in Fig. 3.2.11.

MUD50 staining of immunoblotted WS584 sheath proteins confirms the result shown in Fig. 3.2.1b, where a crude sheath preparation was used. The sheath of this isolate lacks all of the wild type sheath proteins recognised by MUD50 (Fig. 3.2.11a, lane 3) but has a small number of proteins of similar MW to the wild type; in particular

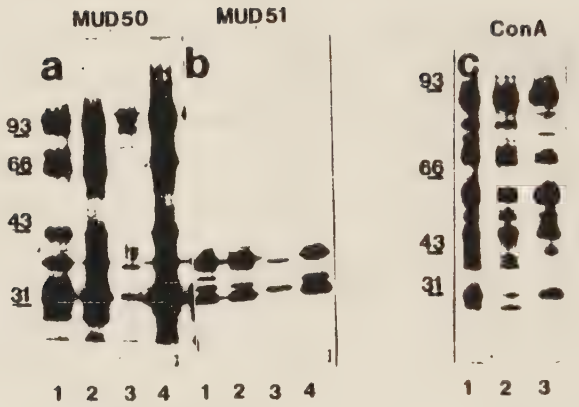


Figure 3.2.11: Immunoblots (a & b) and ConA-peroxidase staining (c) of wild isolate sheath. Lanes in (a & b) : (1) NC4; (2) NP84; (3) WS584; (4) WS472 stained with (a) MUD50 and (b) MUD51. Lanes in (c): (1) NP84; (2) WS472; (3) WS584. 10% acrylamide resolving gel.

proteins of ca. 33kd and 39kd. Both these proteins were also stained by MUD51 (Fig. 3.2.11b, lane 3), suggesting they may be analogous to ShA and the 40kd proteins. These proteins were also stained by MUD52 (data not shown). The 34kd sheath protein was not stained.

ConA-peroxidase staining of WS584 sheath proteins did not reveal the same degree of polymorphism as the mAb's (Fig. 3.2.11c, lane 3) although there were clearly differences to the wild type (Fig. 3.2.11c, lane 1).

A fourth type of protein polymorphism which was not described in 3.2.1 is exhibited by WS472. This polymorphism results in the loss of two MUD50 proteins which migrate slightly slower than BSA (66kd, Fig. 3.2.11a, lane 4 compared to lanes 1 & 2) and the appearence of a band in an intermediate position. No change was seen in the pattern of MUD51 staining (Fig. 3.2.11b, lane 4) and there were only minor changes in the ConA-peroxidase staining of this wild isolate (Fig. 3.2.11c, lane 3). Neither WS472 or WS584 were characterised genetically. This was attempted for WS584 but no WS584 x wild type diploids have so far been obtained.

Of the polymorphic wild isolates found, only WS584 was noticeably different phenotypically. Slugs of this strain were much smaller the other wild isolates' slugs and showed a marked tendency to cease migration and form fruiting bodies. The sheath of WS584 appeared to be thinner than the other wild isolates' and was relatively weak.

CHAPTER 3.3: CELL TYPE SPECIFICITY OF ANTI-SHEATH MONOCLONAL ANTIBODIES

In previous sections it has been shown that the sheath and slug cells share at least one protein and a number of antigenic determinants. The shared protein, PsA, is recognised by MUD1, which has been shown to be specific for prespore cells in the slug (Gregg *et al.*, 1982). It was therefore of interest to compare the cell type specificity of MUD50, which also recognises PsA.

3.3.1 Immunoblots of Anterior and Posterior Slug Cells

Prestalk and prespore cells can be separated by cutting slugs at the supposed prestalk/prespore boundary (see Chapt. 1). This will not yield pure populations of each cell type, partly because it is not possible to accurately assess the position of the boundary and also because of the presence of prestalk-like cells in the prespore region (Sternfeld & David, 1982). However, one of the strains used in this work has a very high proportion of prestalk cells (NP84, which has ca. 35% prestalk, Voet *et al.* 1983; see also 3.3.3). This makes it much easier to obtain relatively pure prestalk cells. Immunoblots of equal numbers of anterior and posterior cells from NP84 and WS576 (the PsA of which is polymorphic and can, therefore, be unambiguously identified) are shown in Fig. 3.3.1.

MUD1 staining shows that for WS576 the posterior cell extract is highly enriched for prespore cells since PsA is

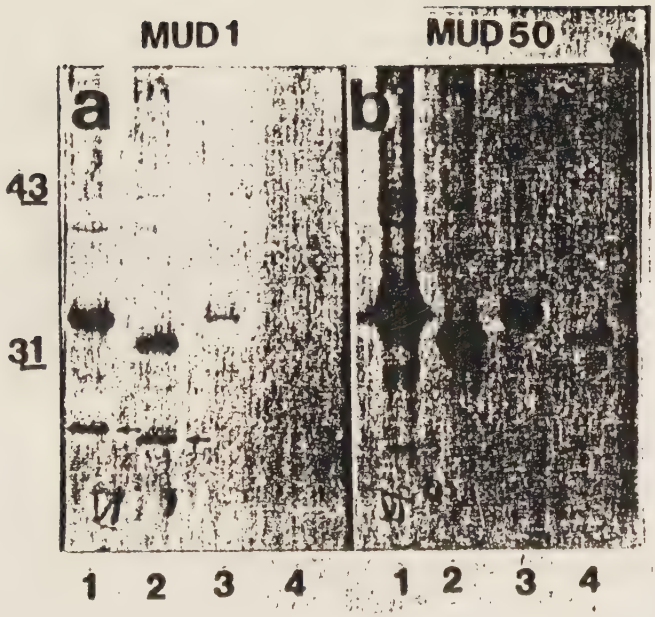


Figure 3.3.1: Immunoblots of anterior and posterior slug cells stained with (a) MUD1 and (b) MUD50. Lanes: (1) WS576, posterior; (2) NP84, posterior; (3) WS576, anterior; (4) NP84, anterior. Equal numbers of cells were loaded to each lane. 12% acrylamide resolving gel.

heavily stained in this (Fig. 3.3.1a, lane 1) but not in the anterior (Fig. 3.3.1a, lane 3) preparation. Some very light staining of the anterior cell immunoblot did occur with MUD1 but was most probably due to contamination with a small number of prespore cells (Fig. 3.3.1a, lane 3). For NP84, MUD1 staining was entirely restricted to the prespore immunoblot (Fig. 3.3.1a, lanes 2 & 4). The lower MW proteins which may be related to PsA were clearly stained in this figure (small arrows).

MUD50 staining is generally more sensitive than MUD1 staining, so that when the same anterior cell preparations as in Fig. 3.3.1a were immunoblotted and stained with MUD50 significant levels of staining were seen (Fig. 3.3.1b, lanes 3 & 4), particularly in WS576. Posterior cell extracts stained heavily with MUD50 (Fig. 3.3.1b, lanes 1 & 2). The MUD50 staining in this case was not restricted to PsA but other proteins as well, suggesting that all slug cell proteins recognised by MUD50 are prespore cell specific.

3.3.2 Monoclonal Antibody Staining of Slug Frozen Sections

In order to confirm the MUD50 prespore cell specificity, and to determine whether the proteins recognised by MUD50 were on the cell surface or intracellular, frozen sections of slugs were stained with each of the mAb's (the sections were a gift from Prof. J. Gregg).

The distribution of mAb staining within the slug was the same for MUD1, MUD50, MUD52 and MUD54; all were prespore

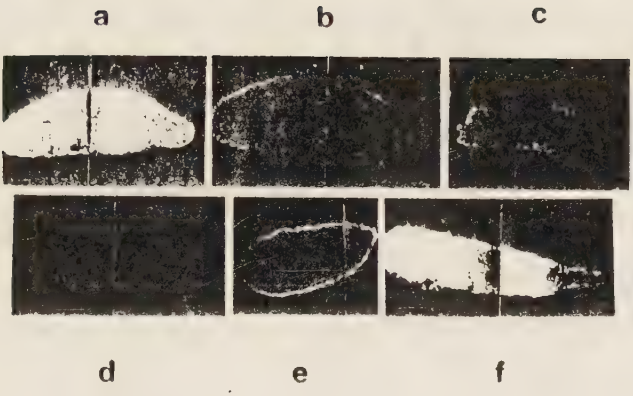


Figure 3.3.2: mAb staining of frozen sections of slugs (a) MUD50; (b) MUD51; (c) MUD52; (d) MUD53; (e) MUD54 (f) MUD1. In all cases the slug tip is pointing to the right of the figure. (Sections prepared by Prof. J. Gregg).

cell specific (Fig. 3.3.2) although the staining intensity varied widely between mAb's (the prespore specificity of MUD52 and MUD54 is difficult to discern). Unstained prestalk-like cells are clearly seen in the prespore region of slugs stained with MUD1 and MUD50. For MUD1 and MUD50 at least, the staining was at the cell surface.

As expected from their lack of reaction with slug cell proteins on immunoblots, MUD51 and MUD53 did not stain the slug sections at all. The fixation procedure used to obtain good mAb staining of the cell surface in these sections apparently destroys the sheath (the apparent staining of the sheath with MUD54 is an artifact; staining was often seen at the edges of sections).

3.3.3 Flow Cytometry of Slug Cells With MUD50

The location of the MUD50 antigenic determinant at the cell surface makes it feasible to use this mAb for flow cytometry in a FACS-IV cell sorter. For FACS analysis, slugs are dissociated to single cells by mild protease treatment, reacted with mAb and fluorescently labelled anti-mouse Fab'2 and then scored for mAb binding (fluorescence intensity) and cell size (light scatter). The cells are thus distinguished on the basis of mAb reactivity and cell size (see Krefft et al., 1983b and Voet et al., 1983). The data presented below was obtained by M. Bruederlein and analysed according to Voet et al., 1983).

The results of FACS analysis of NP84 with MUD1 and MUD50

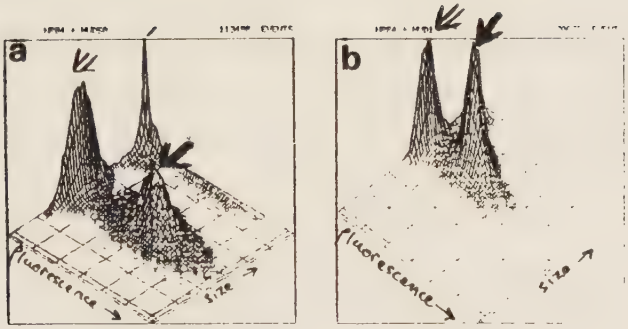


Figure 3.3.3: Flow cytometry (FACS-IV) analysis of slug cell type proportions using (a) MUD1 and (b) MUD50. (The data in this figure was obtained by M. Bruederlein).

are shown in Fig. 3.3.3a & b respectively. The large peak in the middle of each figure (solid arrow) is prespore cells (small and fluorescently labelled) and the peak along the size axis (open arrows; large, unlabelled cells) is prestalk. This demonstrates that MUD50 is specific for prespore cells (or at least for their surfaces). Some differences can be seen between the labelling of slug cells with the two mAb's. For example, the MUD50 prespore peak covers a wider range of fluorescence intensities than the MUD50 peak. However, both mAb's recognise the same proportion of slug cells (65%, see Krefft et al., 1983b) and recent double-labelling experiments have demonstrated that there is complete overlap between MUD1 and MUD50 labelling, ie. they recognise the same cells (Voet, personal communication).

The preceding 3 sections suggest, therefore, that PsA and the other MUD50 (and MUD52 & MUD54) recognised slug cell proteins are found only in or on the surface of prespore cells in the slug ie. that the slug cell/sheath overlap in antigenic determinants and specific proteins is restricted to prespore cells.

3.3.4 Monoclonal Antibody Reactivity in Fruiting Bodies

Given the cell type specificity of the mAb's in the slug and the probable precursor-mature cell relationship between the cell types in the slug and the mature fruiting body (see Chapt 1), a comparison of sheath, slug cells (ie. prespore cells) and mature cell types was made. As in preceding sections, NP84 and a polymorphic wild isolate were analysed in parallel to aid in the identification of PsA.

(a) Spores: Mature spores of NP84 and Dd61 were separated into spore "cell" (SDS soluble) and spore "coat" (β-mercaptoethanol soluble; Wilkinson & Hames, 1983) proteins and immunoblotted (Fig. 3.3.4, lanes 3 & 4 respectively). Comparison of slug cell and spore cell immunoblots stained with MUD50 (Fig. 3.3.4A, lanes 2 & 3) and MUD1 (Fig. 3.3.4C, lanes 2 & 3) shows that PsA and some other prespore proteins are present in the mature spore cell (no MUD51 staining of either was seen, Fig. 3.3.4B, lanes 2 & 3).

The spore coat also contained PsA but few, if any, other prespore cell proteins and no MUD51 stained proteins (Fig. 3.3.4A-C, lane 4). Other spore coat proteins may have been present but undetected. Relatively low amounts of protein had to be loaded in the spore coat lanes because the spore coat extract contains material which reacts strongly with peroxidase-DAB in the final staining step, giving rise to a large number of bands in the absence of antibody. This was avoided in Fig. 3.3.4 by applying small amounts of spore coat extract.

No spore specific proteins ie. proteins found in the mature spore but not in prespore cells, were detected and the only case of mature spore/sheath overlap was the PsA protein (compare lanes 1, 3 & 4, Fig. 3.3.4).

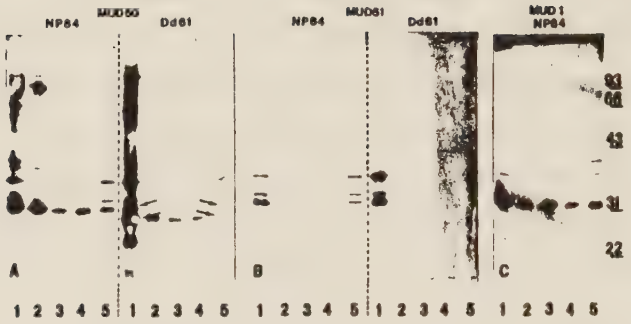


Figure 3.3.4: Cell type distribution of antigenic determinants and proteins recognised by mAb's, stained with (A) MUD50, (B) MUD51 and (C) MUD1. Lanes: (1) sheath; (2) slug cells; (3) spore cells; (4) spore coats; (5) stalks. See the text for a definition of spore cells and spore coats. 12% acrylamide resolving gel.

(b) Stalks: The pattern of mAb recognition of stalk proteins is strikingly similar to that of the sheath (Fig. 3.3.4A-C, lanes 1 & 5; the arrows in Fig.3.3.4A indicate the positions of PsA and ShA). In particular, it is the only "tissue" other than the sheath which reacts with MUD51. All of the major sheath proteins (PsA, ShA, 34kd and 40kd) are present plus some of the higher MW MUD50 recognised sheath proteins. Most of the proteins common to the sheath and stalk are cellulase sensitive in the sheath (see 3.1.2).

The presence of PsA in the stalk is rather surprising (lower arrow, Fig. 3.3.4A, lane 5 of Dd61) since this protein is found in prespore but not prestalk cells in the slug.

3.3.5 The Sheath and Secreted Proteins

Proteins destined for the sheath must be secreted by slug cells. Landfear and Lodish (1980) have demonstrated that disaggregated developing cells continue to synthesise the proteins characteristic of the stage of development reached at the time of disaggregation when shaken in suspension under appropriate conditions. As an extension of the cell type specificity studies described above, slug cells were disgaggregated (by mild protease treatment) and shaken in suspension in the presence and absence of cAMP (Landfear & Lodish, 1980). Samples of the suspension buffer were withdrawn at various times and probed with the mAb's.

In the competition ELISA assay, material capable of inhibiting the ELISA of MUD50 but not MUD51 was secreted by

disaggregated slug cells (Fig. 3.3.5). The inclusion of cAMP in the suspension buffer had no effect on the amount of material secreted. The ELISA of MUD52 was also inhibited by this material (data not shown).

Immunoblots of the secreted material showed that the major mAb recognised protein secreted was PsA (Fig.3.3.6a, b, c). The identification of the protein as PsA is unambiguous because

(a) the secreted protein is polymorphic (compare lanes 1, 2, & 3 in Fig. 3.3.6a & b) and,

(b) this protein is not recognised by MUD52, which recognises ShA but not PsA (see Fig. 3.2.4b). No staining at all of immunoblotted secreted proteins was seen with MUD51 (data not shown).

It appears, therefore, that the sheath specific antigenic determinants defined by MUD51 and MUD52 are not secreted by disaggregated slug cells ie. the secretion of PsA and these determinants is under separate control. The possibility that disaggregation affects the addition of these determinants but not the secretion of the proteins which normally carry them cannot be excluded. In this case, the (unmodified) proteins would not be detected by the mAb's.

The data presented in this chapter is summarised in Table 3.3.1.

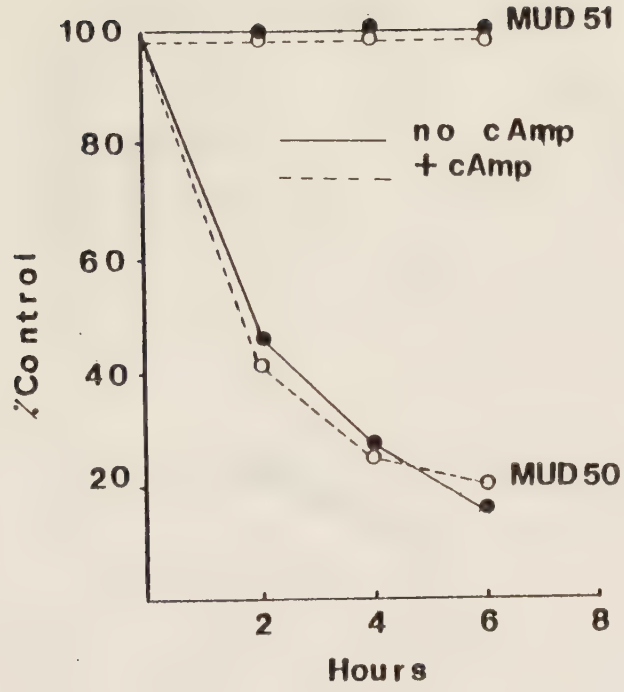


Figure 3.3.5: Competition ELISA of mAb determinant secretion by disaggregated slug cells. Open circles, dashed lines: cAMP added at 2 h intervals to a final concentration of 100μM after each addition. Closed circles, solid line: no additions.

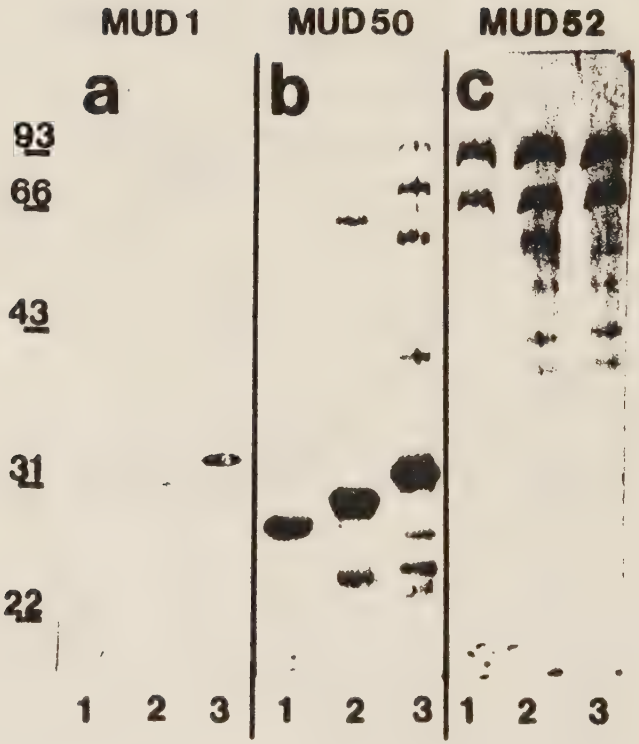


Figure 3.3.6: Immunoblots of proteins secreted by disaggregated slug cells during 4 h in suspension (no additions to suspension buffer) stained with (a) MUD1, (b) MUD50 and (c) MUD52. Lanes: (1) Dd61; (2) NP84; (c) WS576. 12% acrylamide resolving gel.

Table 3.3.1: Summary of cell type specificities data. Sp coat is spore coat etc. *, see Table 3.1.1; #, see Fig. 3.3.4; ^, see Fig.3.2.6. Since the results were the same, the sheath and stalk data for MUD51 and MUD52 have been presented together.

mAb	Sheath	Slugs	Sp cells	Sp coat	Stalk	Secreted
MUD1	PsA	PsA	PsA	PsA	PsA	PsA
MUD50	PsA ShA 34kd 40kd others*	PsA + others*	PsA + others#	PsA alone?	PsA ShA 34kd	PsA + others^
MUD51/ MUD52	ShA 34kd 40kd	none some*	none some#	none none	ShA 34kd 40kd	none some^

CHAPTER 3.4: PRELIMINARY STUDIES OF SHEATH FUNCTION

Proposed functions of the sheath were divided into three broad categories in Chapt. 1:

(a) the role of the sheath in the decision whether to form a migratory slug or a fruiting body; the slug-fruit switch

(b) the role of the sheath in establishing and/or maintaining the correct prestalk/prespore proportions and pattern

(c) the role of the sheath in slug migration.

This chapter describes some preliminary experiments dealing with the role of the sheath in the slug-fruit switch and proportion regulation. The experiments were not exhaustive, but aimed to show that the partial characterisation of the protein component of the sheath could be used as a starting point for functional studies. Some work which was done in collaboration with Drs. E. Smith and P. R. Fisher and is relevant to this Chapter is included as an Appendix.

3.4.1 The Role of the Sheath in the Slug-Fruit Switch

While engaged in the work on slug speeds (see Appendix), it was noted that for each particular strain a reproducible proportion of slugs formed fruiting bodies rather than resuming migration following transfer. In all cases, smaller slugs showed a much greater tendency to form fruiting bodies than larger slugs. This size-dependent fruiting body formation was more frequent and affected larger slugs when

transfer was to charcoal containing agar rather than normal non-nutrient agar. The sheath of slugs on charcoal agar is different in appearence to and weaker than the sheath of slugs on normal agar, raising the possibility that the charcoal induction of fruiting body formation may be related to changes in the sheath.

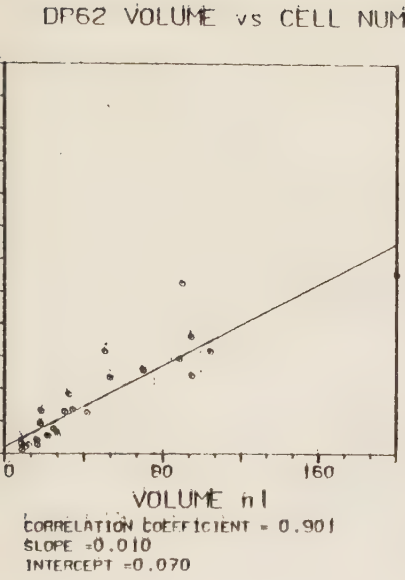
The nature of the charcoal effect is not known. Charcoal adsorbs many molecules, so that the effect on the slug-fruit switch may be due to adsorption of a migration inducing molecule and be unrelated to the effect on the sheath. Unlike charcoal, the effect of cellulase on the sheath *in vitro* is known and, since the enzyme degrades one of the major components of the sheath (ie. cellulose), it may have a similar weakening action.

The experiments described below were performed using strain DP62, the isogenic diploid of haploid strain NP73 (the strain from which NP84 was derived). An isogenic diploid is obtained via short term treatment of haploids with a lethal concentration of a microtubule inhibiting compound (eg. thiabendazole) which leads to chromosome doubling (Welker & Williams, 1980). This results in a diploid with the same genotype but twice the ploidy of its haploid parents. The principal difference between an isogenic diploid and its haploid parent is that the diploid's cells and spores have approximately twice the volume as the haploid's (Stenhouse & Williams, 1981). As a consequence of this difference, a haploid slug of a given size should contain twice as many cells as an equivalent diploid slug. This was confirmed for slugs of DP62 and NP73

(Fig. 3.4.1), although when other haploid-isogenic diploid pairs were examined there was some deviation from the expected 2:1 ratio of cells per unit slug volume.

When DP62 slugs were transferred to non-nutrient agar, all of the smallest size class of slugs formed fruiting bodies within 20h, as did a small proportion of larger slugs. The others continued to migrate (Fig. 3.4.2, control). If slugs were transferred to agar plates which had been previously spread with 0.2ml of a 5 mg/ml cellulase solution, more slugs in the larger size classes formed fruiting bodies than in the control. As the cellulase concentration was increased, the percentage of fruiting bodies formed also increased as slugs of the larger size classes formed fruiting bodies, so that at 20 mg/ml cellulase in the spreading solution (ie. 4mg of cellulase added per 30ml plate) more than 50% of slugs had formed fruiting bodies after 20h.

A second effect of charcoal agar on slugs is that the average speed of slug migration is significantly faster on charcoal than on normal agar (see Appendix). No change was seen in the speed of the slugs which continued migration in the experiments described in Fig. 3.4.2. Changes of the same order as those seen on charcoal should have been detectable.



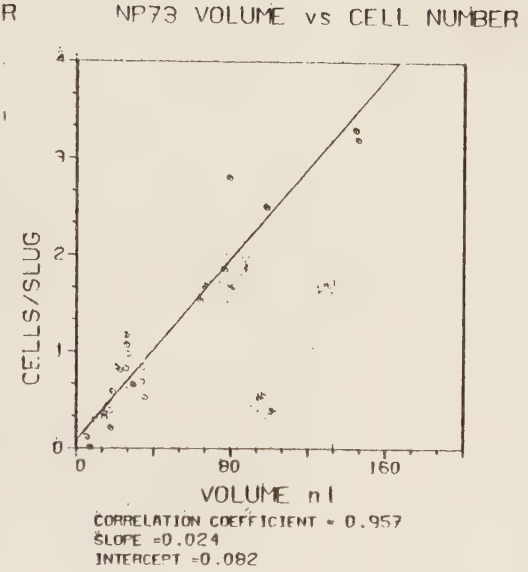


Figure 3.4.1: The relationship between slug size (volume, nl) and numbers of cells (x 10^5) per slug. Volume was estimated from the area of each slug (see Appendix) by assuming that the slug is approximately cylindrical. Linear regression data is included on the figure. Note that the ratio of diploid : haploid slope (ie. cells per unit volume) is ca. 2 : 1.

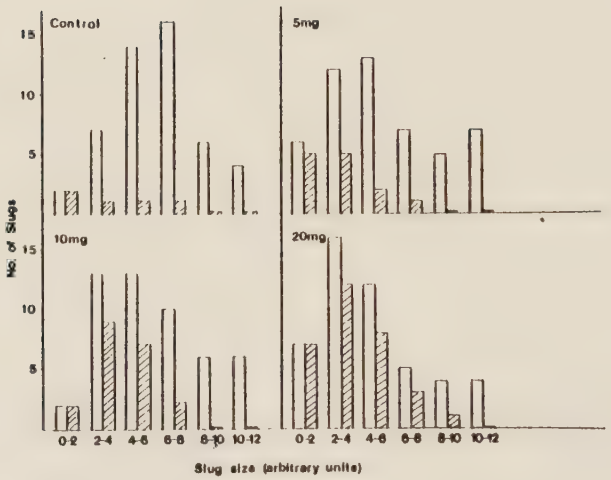


Figure 3.4.2: Histogram of the slug size (area in arbitrary units, see Appendix) and cellulase concentration dependence of cellulase induction of fruiting body formation. The shaded columns represent slugs which formed fruiting bodies within 20 h of transfer to non-nutrient agar plates spread with 0.2ml of cellulase solution (which was allowed to dry before slug transfer). The concentration of the solution (mg/ml) is given in each panel of the figure. The unshaded columns are the total numbers of slugs in each size class.

Cellulase can, therefore, mimic the effect of charcoal on the slug-fruit switch but not on slug speed.

3.4.2 The Effect of Cellulase on Sheath Proteins *in vivo*

When the sheath of NP84 and WS576 slugs which had migrated on cellulase-containing agar (ie. the cellulase was included in the agar rather than spread on the surface) was collected, immunoblotted and stained with MUD50 or MUD51, several changes in the sheath proteins were observed. (Note: the proteins referred to here are those which can be extracted by SDS from the sheath of slugs which have migrated on cellulase-containing agar, not proteins which are released from the sheath by cellulase *in vitro*, see 3.1.2). The inclusion of the cellulase *in* the agar removes the uncertainty concerning the actual concentration of enzyme seen by the slugs in the experiments described in the preceding section.

At both 0.1 mg/ml and 1.0 mg/ml cellulase in the agar, ShA and the 34kd and 40 kd proteins were missing from the sheath (Fig. 3.4.3a & b, lanes 3, 4 & 7, the solid arrows indicate the expected positions of the 40kd and ShA proteins). The loss of ShA, and the lack of MUD51 recognition of PsA, is especially clear when lane 7 in Fig. 3.4.3a & b are compared. MUD50 stained a 34kd protein (WS576's PsA) but no staining of the same material was seen with MUD51.

As a control to see whether the cellulase effect on the sheath proteins may have been due to the presence of protein

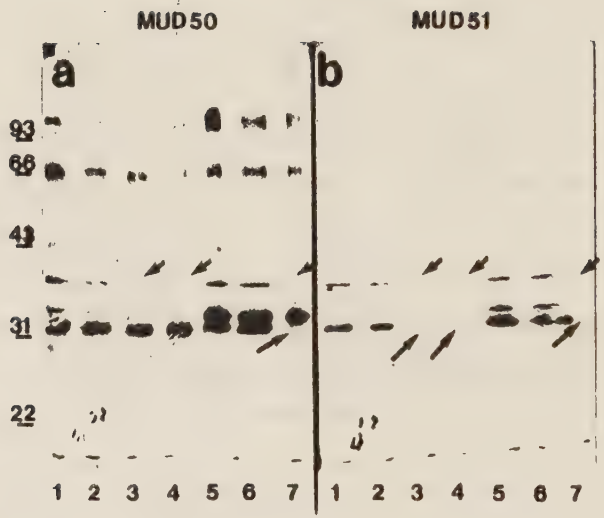


Figure 3.4.3: *In vivo* effects of cellulase on the sheath of slugs which have migrated on cellulase-containing agar; immunoblots stained with (a) MUD50 and (b) MUD51. Lanes: (1) sheath from slugs of strain NP84 migrated on agar containing 0.1mg/ml BSA (control); (2) NP84, 1.0mg/ml BSA; (3) NP84, 0.1mg/ml cellulase; (4) NP84, 1.0mg/ml cellulase; (5) WS576, 0.1mg/ml BSA; (6) WS576, 1.0mg/ml BSA; (7) WS576, 0.1mg/ml cellulase. The arrows indicate the expected positions of the 40kd and PsA proteins in the lanes where they were not stained. 12% acrylamide resolving gel.

(ie cellulase) in the agar rather than due to the enzymatic activity of the cellulase, identical experiments where BSA was included in the agar rather than cellulase were performed. The sheath from these experiments (Fig. 3.4.3a & b, lanes 1, 2, 5 & 6) is normal.

The sheath from slugs on cellulase agar is thinner in appearance and is much weaker than in the control. For example, it was not possible to collect the sheath from WS576 slugs on 1.0 mg/ml cellulase because it was too weak to pick up. Despite this weakening of the sheath, neither NP84 nor WS576 showed an increased tendency to form fruiting bodies at these (or higher) cellulase concentrations. This is in contrast to DP62, which could be induced to fruit by cellulase. It is also in contrast to the fruit induction on charcoal, which affects NP84 (data not shown). The difference in susceptibility to cellulase induction of fruiting body formation between NP84 and DP62 is not due to differences in the way in which the cellulase was applied (in the agar rather than on the surface), since NP84 also continued migraton on plates spread with 20 mg/ml cellulase.

3.4.3 Reexamination of *in vitro* Protein Release by Cellulase

The data initially presented for cellulase release of sheath proteins was obtained using strain NP84, where no clear cut distinction between PsA and ShA can be made. In view of the selective loss (*in vivo* release ?) of ShA from the sheath of slugs migrated on cellulase, the cellulase release of sheath proteins *in vitro* was re-examined using a polymorphic wild isolate (WS576). Fig. 3.4.4 presents a

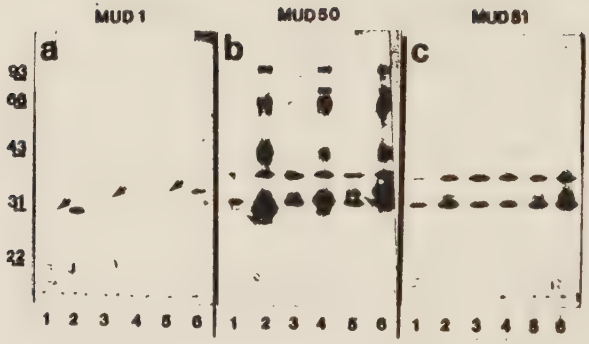


Figure 3.4.4: Comparison of *in vitro* cellulase and SDS release of sheath proteins. Immunoblots stained with (a) MUD1, (b) MUD50 and (c) MUD51. Lanes: (1) Dd61, cellulase release; (2) Dd61, SDS release; (3) NP84, cellulase release; (4) NP84, SDS release; (5) WS576, cellulase release; (6) WS576, SDS release. The arrows indicate the expected position of PsA in the lanes where it was not stained. 12% acrylamide resolving gel.

comparison of SDS and cellulase release of sheath proteins.

MUD1 stained immunoblots of cellulase and SDS released proteins clearly show that PsA is not released from the sheath by cellulase (Fig. 3.4.4a, lanes 1, 3 & 5; the arrows indicate the expected position of PsA in the cellulase extracts). This can also be seen in the polymorphic wild isolates stained with MUD50 (Fig. 3.4.4b, lanes 1 & 5) but not in the NP84 MUD50 stained cellulase extract (Fig. 3.4.4b, lane 3). The identity of the cellulase released protein as ShA is confirmed by the ability of MUD51 to stain it in cellulase extracts (Fig. 3.4.4c, lanes 1, 3 & 5).

The *in vitro* and *in vivo* cellulase data are in agreement. *In vivo*, ShA, the 34kd and 40kd proteins are lost from the sheath and *in vitro* the same proteins are released from the sheath. ShA can, therefore, be referred to as a cellulase sensitive protein and PsA as a cellulose resistant protein.

3.4.4 The Effect of Cellulase on Cell Type Proportions

It is possble that the cellulase effect on the slug-fruit switch is due to an increase in the rate of diffusion of a migration inducer out of the slug (see Chapt. 4). Since it has been proposed that small diffusible molecules may be involved in proportioning in D.discoideum (see Chapt. 1), it was decided to examine the cell type proportions in cellulase-treated slugs. The results are presented in Table 3.4.1.

Table 3.4.1: Cell type proportions in cellulase treated slugs. Slugs of strain NP84 were transferred to Noble agar plates with or without cellulase, allowed to migrate for 20h and then the % prespore cells determined by flow cytometry as in 3.3.3 using MUD50 as the labelling mAb.

Cellulase mg/ml	% Prespore
0 (control)	63.0
0.1	62.6
1.0	65.1

As can be seen from the Table, cellulase had no detectable effect on the proportion of prespore cells in the slug.

CHAPTER 4: DISCUSSION

4.1 The Sheath Proteins

The work described in this thesis confirms and considerably extends the work of Smith and Williams (1979). The existence of sheath associated proteins is unequivocally demonstrated. Quite surprisingly, a considerable degree of sheath/prespore cell, sheath/stalk and a lesser degree of sheath/spore sharing of particular proteins was also demonstrated. These latter observations raise questions concerning the function and sites of synthesis of sheath proteins (see 4.5).

The proteins detected by the mAb's are restricted to the developmental phases of the life cycle. MUD50 and MUD52 recognise carbohydrate antigenic determinants (3.1.4), so their exclusive reactivity with the developmental phase may reflect the marked differences in protein glycosylation between vegetative and developing cells (Ivatt et al., 1981). In addition, since these two mAb's recognise all of the proteins detected by the other mAb's (MUD1 and MUD51), all the proteins discussed here are glycoproteins.

The conditions required for the release of sheath proteins (SDS, urea, and also guanidine-HCl) suggest that protein conformation and possibly protein-protein interactions are responsible for the retention of many proteins in the sheath. This is in contrast to the work of Freeze and Loomis (1977a, see 1.4), who found a minor proteinaceous fraction which was apparently covalently linked to cellulose. The relationship between Freeze and

Loomis's material and the proteins reported here is unclear.

Cellulase enzyme releases a subset of the sheath proteins *in vivo* and *in vitro* (ShA but not PsA, plus at least the 34kd and 40kd sheath proteins). Sheath proteins can, therefore, be divided into two groups: cellulase-sensitive (ie. those released by cellulase) and cellulase-insensitive (not released by cellulase).

This division must reflect a difference in the way in which these proteins are retained in the sheath. Cellulase-sensitive proteins apparently rely on cellulose-protein interactions for their retention ie. they are cellulose associated. In terms of Hohl and Jehli's description of the sheath, the "amorphous protein containing matrix", which remains intact after cellulase digestion (Hohl & Jehli,1973), contains the cellulase-resistant fraction, while the cellulase-sensitive proteins are associated with the cellulose fibrils. The extensive overlap between sheath cellulase-sensitive proteins and stalk proteins, as opposed to the lack of cellulase-sensitive/spore coat protein overlap (3.3.4), supports this proposal (see 4.4).

The extensive antigenic overlap between ShA, the 34kd and 40kd proteins (all of which are cellulase-sensitive) is consistent with some functional relationship between them. These proteins are recognised by MUD50, MUD51, and MUD52 (3.1.3) and so share at least 3 antigenic determinants. Two of the antigenic determinants (MUD50 and MUD52) are carbohydrate and are found on other sheath (and cellular proteins), but the MUD51 determinant is clearly sheath and stalk specific (3.3.4). Antigenic determinants are three

dimensional structures, often involving groups of residues well separated in the protein's primary structure (Todd et al., 1982; Berzofsky et al., 1982; East et al., 1982), so the sharing of 3 antigenic determinants implies a high degree of structural similarity. Such mAb structural similarity has been shown to be correlated with functional similarity in a number of cases (Pruss et al., 1981; Glenney et al 1982; Gown & Vogel, 1982) and illustrates the usefulness of mAb's as probes of protein structure. The shared "function" in this case is the proposed cellulose association of the cellulase-sensitive proteins.

HPLC peptide mapping of the 32kd and 40kd proteins from the sheath of strain NP84 suggests that the structural similarity implied by the antigenic overlap between these two proteins does exist. The interpretation of these results is complicated by the subsequent observation that the 32kd "protein" from the sheath is composed of a mixture of the PsA and ShA proteins, possibly in a ca. 2:1 ratio (based on staining intensity in Fig. 3.2.6). However, the PsA and ShA proteins are probably themselves closely related.

This is based partly on antigenic and biochemical considerations (see 4.2) and on the reasoning that if PsA and ShA were markedly different, then the virtually complete overlap in peptide R_f's between the deglycosylated peptide maps of the PsA/ShA and 40kd proteins would imply that *one* protein (the 40kd) could be related to *two* unrelated proteins (if PsA and ShA are unrelated). Such a relationship is clearly illogical.

Consequently, the conclusion that the 40kd is related to

either ShA or PsA, which are in turn related to each other, is not unfounded. It is clear, however, that more complete peptide mapping studies or gene sequence data are required to fully resolve this question.

From a technical viewpoint, the HPLC data presented here demonstrate the utility of post-cleavage deglcosylation for preparing peptide maps of glycoproteins which cannot be easily obtained in pure form. The need to deglycosylate at some stage is illustrated by the effect of HF treatment on the R_t's of peptides from ovalbumin and the 40kd protein.

One proviso must, however, be made. In many cases sugar groups provide protection against proteolytic cleavage (see Gahmberg, 1981), so that cleavage prior to deglycosylation, as was performed here, may be incomplete or blocked entirely at particular "protected sites" compared to post-deglycosylation cleavage. Biosynthetic differences in glycosylation of proteins related at the primary sequence level could, therefore, result in differences in the relative amounts of peptides generated by proteolysis. This may partly explain the quantitative differences between the qualitatively almost identical deglycosylated peptide maps of the 40kd and PsA/ShA proteins (eg. the peaks at R_t = 51 and 55 min. in Fig. 3.1.8b).

4.2 The Relationship Between PsA and ShA

The existence of polymorphisms at the *psA* locus allowed the PsA and ShA proteins to be distiguished from each other (3.2.2). When viewed as a whole, their common MW in the wild type, similarity in pI, resolution into two or more

similarly spaced spots in the isoelectric dimension of 2-D gels (see Fig 3.2.6) and possibly common relatedness to a third protein (the 40kd sheath protein, 3.1.6) all suggest that PsA and ShA are closely related.

Their differences in mAb reactivity, cellulase sensitivity, cellular distribution, and possible differences in regulation of their secretion (see 4.5) may be due to differences in function (eg. PsA is able to form intermolecular disulphide bridges and ShA is able to associate with cellulose fibrils, see 4.4).

Some parallels can be drawn between PsA and ShA and the two endogenous D. discoideum lectins, discoidins I and II (although it is clear that the discoidins and PsA and ShA are different, 3.1.2). These lectins (see Barties & Frazier, 1982) have overlapping but not identical sugar specificities and amino acid compositions and share a number of peptides and antigenic determinants (Frazier et al., 1975; Berger & Armant, 1982) ie have a similar degree of overlap as PsA and ShA. They differ markedly in regulation (Frazier et al., 1975; Ma & Firtel, 1978), are encoded by separate genes (Ma & Firtel, 1978) and are probably localised in different cell types (Barondes et al., 1983; but see Lam et al., 1981) ie. they differ in similar ways as PsA and ShA.

4.3 Antigenic and Protein Overlap Between the Sheath and Prespore Cells in the Slug

In slugs, the sheath/prespore cell overlap is on two levels: the sharing of antigenic determinants and the sharing of specific proteins.

On the antigenic level, there is an extensive sharing of carbohydrate determinants which involves a large number of proteins, many of which are detected only in the sheath or in slug cells (Table 3.1.1). A single protein determinant (defined by MUD1) is also shared. The cell type specificity of MUD50 and MUD52 may be analogous to the cell type specificity of [^{3}H]-fucose incorporation since both are restricted to prespore cells in the slug (Gregg & Karp, 1978; Lam & Siu, 1981).

The second level of overlap is the sharing of specific proteins. The best defined example of this is the PsA protein, although there are probably other examples (see 3.1.3)

In the case of proteins carrying the antigenic determinants defined by MUD50 and MUD52 (which are carbohydrate) it is unclear whether the cell type specificity of the proteins recognised by these mAb's is at the level of the polypeptides themselves, which implies cell type specific gene expression, or whether the polypeptides are synthesised in all slug cells but only glycosylated with the MUD50 and MUD52 determinants in prespore cells. This second possibility implies a cell type specific difference in glycosylation of specific polypeptides. While such a difference has not been reported in D.discoideum, tissue and developmental stage differences in the glycosylation of specific polypeptides have been reported for a variety of T-lymphocyte proteins (see Pink, 1983) and a mouse differentiation antigen (Rougon et al., 1982).

However, the ease with which cell type specific mRNA's

can be detected (Barklis & Lodish, 1983) and the lack of any direct evidence for differential glycosiation suggests that regulation of gene transcription is the most likely means used by this organism to generate cell type specific antigenic differences. The MUD50 and MUD52 prespore specificity probably arises, therefore, via prespore specific synthesis of the proteins involved ie the genes for these proteins are not expressed in prestalk cells (but see 4.5).

4.4 Overlap Between the Sheath and Mature Fruiting Bodies

The overlap between the sheath and mature fruiting bodies is less complex, particularly with the stalk, where the antigenic and protein overlap are almost identical ie. most or all of the proteins recognised by the mAb's in the stalk are also present in the sheath (3.3.4). Virtually all of the sheath/stalk common proteins are cellulase sensitive in the sheath ie. possibly cellulose associated (4.1). Stalk and sheath cellulose are indistinguishable chemically (degree of crystallinity etc., Freeze & Loomis, 1978), very similar ultrastructurally (George et al., 1972) and the sheath and stalk proteins may be synthesised by the same cells (prestalk cells; George et al., 1972; see 4.5). The sheath/stalk protein overlap is, therefore, logical if one accepts the cellulose association of the proteins involved. The presence of the PsA protein (a cellulase-resistant protein) in the stalk is the only exception, although it presumably performs the same function in the stalk as in the sheath.

The spore coat also contains proteins which are

apparently associated with cellulose fibrils (Hemmes et al., 1972). However, there are significant differences between the spore coat and the sheath and stalk. Spore coat proteins are held in place by disulphide bonds (Orlowski & Loomis, 1979; Wilkinson & Hames, 1983) whereas the sheath (and stalk) proteins are not (3.1.1). This, plus the ultrastructural differences between spore coats and the sheath (and stalk; Hemmes et al., 1972), the specialised function of the spore coat (protection of the spore cell) and the probable difference in the sites of synthesis of spore coat (prespore cells) and the stalk and sheath (prestalk cells, see 4.5) may explain the absence of cellulase sensitive sheath proteins from the spore coat.

The overlap between the sheath and spore coats detected in this work is restricted to the PsA protein, which is cellulase resistant in the sheath. PsA is almost certainly the same as the 33kd spore coat protein reported by Wilkinson and Hames (1983). For example, both are resolved into multiple spots in 2-D electrophoresis and are the only proteins in this MW region detected in spore coat extracts. Other sheath/prespore/spore coat overlap not detected by these mAb's may also exist, since Takeuchi's anti-spore serum, which reacts with spore coat proteins other than PsA as well as the spore coat acid mucopolysaccharide (Takeuchi,1963; Devine et al.,1983), also reacts with the sheath (Tasaka & Takeuchi, 1979). The proteins recognised by this serum are disulphide linked in the spore coat and prespore specific in the slug ie. similar to PsA rather than ShA (Devine et al., 1983).

Thus, the PsA protein is found in the sheath and the stalk, where it is in a presumably hydrophilic environment and is easily extracted; it is found on the surface of slug (and spore ?) cells, where it may be in a hydrophobic environment; and it is found in the spore coat, where at least some of it is held in place by disulphide bonds and can only be extracted in the presence of reducing agents. How it "knows" which environment it is in at a given time and how it regulates the way it is retained are interesting protein biochemical questions. An important related question is whether the protein is performing the same function in all its various locations, given the differences between them.

Not all spore associated PsA is, however, disulphide linked; some can be extracted by SDS alone. It is not possible to determine whether the unlinked PsA is in the spore coat or whether it is associated with the spore cell. Prespore proteins other than PsA detected by the mAb's are also extracted by SDS alone and so may be associated with mature spore cells. This, in conjunction with numerous other reports of the persistence of prespore proteins in the mature spore (see 1.3), supports the view that there is a direct relationship between prespore differentiation and the differentiation of the mature spore. The relationship between prestalk and mature stalk is uncertain (see Morrissey, 1982; Borth & Ratner,1983). Moreover, many cells which have been characterised as prestalk cells in the slug are actually the anterior-like cells (found in the prespore region) whose role in fruiting is disputed (Sternfeld & David, 1982).

4.5 Possible Sites of Sheath Synthesis

It is clear that some components of the sheath, especially cellulose, must be synthesised by prestalk cells because the tip of the slug is always covered (only prestalk cells are found at the tip). Fibrils, presumably of cellulose, can be seen at the tip, (Claviez, personal communication). In addition, prestalk, but not prespore, cells about to begin fruiting body formation synthesise the cellulose fibrils which form the outermost layer of the stalk (Raper & Fennell, 1952; George et al., 1972). These cells also synthesise the cellulose component of the sheath at this stage (George et al., 1972). It is not known whether prespore cells also synthesise cellulose.

The distribution of different sheath proteins discussed above allows some speculation concerning possible sites of their synthesis. The sheath/stalk specific proteins (ShA, 34kd and 40kd, which are all cellulase sensitive) are possibly synthesised by prestalk but not prespore cells because they are found in mature stalks but not mature spores (since prespore cells and mature spores are very similar in their complements of mAb detected proteins, 3.3.4). The failure to detect the antigenic determinants associated with these proteins in prestalk cell extracts is explained if one assumes they are rapidly secreted to maintain the high rate of sheath synthesis required by migrating slugs.

Indirect evidence for the rapid secretion of proteins from prestalk cells is provided by the observation that [^{3}H]-fucose is not incorporated *into* these cells but is

clearly in the sheath surrounding them and in mature stalks (Gregg & Karp, 1978). This also extends the analogy between fucose incorporation and the distribution of the MUD50 and MUD52 antigenic determinants (4.3).

The sheath/prespore common proteins (of which at least PsA is cellulase resistant) are unambiguously synthesised by at least prespore cells (3.3.1-3). The possibility that they may also be synthesised by prestalk cells and rapidly secreted rather than sent to the cell surface cannot be excluded but the prespore → spore dogma implies that these proteins are synthesised exclusively by prespore cells. PsA may be an exception, since its presence in the stalk implies it is synthesised by all slug cells, although it is undetectable on the surface of or inside prestalk cells (3.4.1-3).

Since sheath proteins must be secreted, an attempt to test the hypothesis made above was made by examining the proteins secreted by disaggregated slug cells (3.3.5). PsA was the major mAb reactive secreted protein. Surprisingly, no sheath specific antigens were detected, even after the addition of cAMP, which maintains the synthesis of most developmentally regulated proteins (Landfear & Lodish, 1980). Thus, the secretion of PsA (and perhaps other sheath/slug cell common proteins) is not affected by disaggregation but the secretion of ShA and the 34kd and 40kd sheath proteins, or at least the antigenic determinants normally associated with them, is blocked by the same treatment ie. the secretion of these proteins is separately controlled.

The significance of this separation of control is unknown, although it appears as though the block is specific for the cellulose associated prestalk specific (?) proteins. It means, however, that in order to test the proposed cell type specificity of the synthesis of the ShA, 34kd and 40kd proteins it may be necessary to first clone the genes coding for them. It would then be possible to check for the expression of specific genes in cells which had been separated by flow cytometry on the basis of recognition by specific mAb's.

The data and proposals relating to the location, sites of synthesis and secretion of the sheath proteins is summarised in Fig. 4.1.

An obvious consequence of the scheme in Fig. 4.1 is that the sheath covering the prestalk cells will be qualitatively different to the sheath covering prespore cells. It also claims that sheath associated proteins are not synthesised by a yet to be demonstrated population of specific sheath producing cells localised immediately adjacent to the sheath but by cells throughout the slug. This clearly explains the gradient in sheath thickness (Farnsworth & Loomis, 1975) and implies that sheath proteins should be found between slug cells as well as in the sheath. This could even be the correct location of some of these proteins. Detailed ultrastructural studies using the anti-sheath mAb's have been commenced to test these proposals (in collaboration with Dr. W. de Priester, Leiden).

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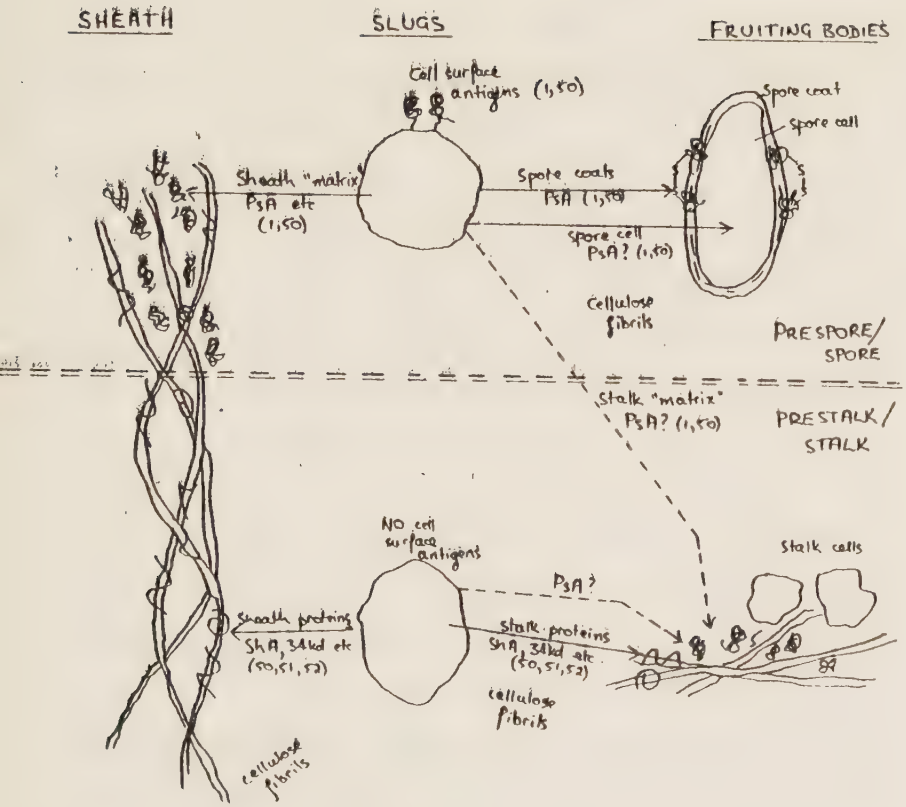

Figure 4.1: Locations and proposed sites of synthesis of proteins recognised by anti-sheath mAb's.

4.6 Polymorphisms of Sheath Proteins

In D.discoideum, polymorphism at the DNA sequence level is very common. For example, the actin, discoidin I, ribosomal RNA and transfer RNA genes all show polymorphism at the DNA sequence level (Kimmel & Firtel, 1982; Welker et al, in preparation) and such polymorphism may be generally very widespread (Ayala, 1983). However, the frequent occurrence of a polymorphism affecting the MW of a specific protein (PsA, 4 of the 6 detected) is surprising.

The regular ±2kd changes in PsA associated with the different *psp*A alleles (which were not, however, associated with detectable changes in pI) are best explained by the addition/subtraction of the same ca. 2kd unit from the wild type 32kd form. This ca. 2kd unit is most likely to be a peptide rather than carbohydrate because:

(a) no changes in any other sheath or cellular glycoproteins were detected in any of the *psp*A polymorphic wild isolates (see Fig. 3.2.6). If the polymorphisms were due to altered glycosylation then these alterations must all be specific for PsA.

(b) a number of lower molecular weight bands which may be partially processed forms of PsA underwent similar changes in mobility. This behaviour of precursor molecules is only expected if the various forms of PsA are processed in the same way ie. do not differ in glycosylation.

(c) all the *psp*A polymorphisms map to a single locus.

At the DNA level these polymorphisms may have arisen via the insertion/deletion of a specific sequence coding for a 12-15 amino acid peptide. The wild type gene must contain at least one such unit, WS576 and HU188 at least two and WS380B and Dd81 none. It is possible, of course, that there may be several such peptides present in the wild type PsA. For example, the circumsporozite surface protein from the sporozite stage of the malarial parasite Plasmodium knowlesi contains a tandemly repeated 12 amino acid peptide which is coded for by a 36 base pair tandem repeat within the gene (Godson et al., 1983). If the PsA protein is directly analogous to the circumsporozite protein ie. contains several of the proposed sequences, then it should show a periodic structure. This is a common feature of many connective tissue proteins (see Godson et al., 1983) and is, therefore, a tempting proposal which can be directly tested.

A quite distinct type of sheath protein polymorphism is exhibited by WS584 (3.2.7). Slug cells of WS584 do not react at all with MUD1, MUD50 or MUD52 and so must completely lack PsA and the MUD50 and MUD52 determinants. The sheath of this isolate is also abnormal in that it reacts with all of the anti-sheath mAb's except MUD1 but shows proteins of 33kd and 39kd as the major sheath proteins and lacks most others.The WS584 33kd and 39kd proteins may be homologous to the wild type ShA and 40kd proteins on the basis of their mAb reactivity. The ConA-peroxidase staining of sheath and slug cell proteins showed fewer changes, suggesting that generalised changes in protein glycosylation are not responsible for the polymorphism. The only conclusion which can be drawn is that the polymorphism seems to principally

affect MUD50 reactive proteins but the precise mechanism is not known.

4.7 Function(s) of the Sheath

(a) The Slug-Fruit Switch: During development, aggregates have the choice between forming a fruiting body or a migratory slug: this choice is termed the slug-fruit switch (1.1 and 1.3).

It is clear that the sheath must be involved in the cellulase induction of fruiting body formation (in the slug-fruit switch) because it is the only cellulose containing "tissue" in the slug and the effect is dependent on cellulase concentration ie. on the degree of cellulose degradation. It is probable that an increase in the rate of diffusion of some compound(s) out of the slug in the presence of cellulase is involved because of the size dependence of the effect (the rate of diffusion from small slugs is greater than from large slugs due to their greater surface area : volume ratio). This provides indirect evidence for the proposed role of the sheath as a barrier to the diffusion of endogenously produced, developmentally relevant molecules.

It is unclear, however, what compound(s) are involved. It is tempting to suggest that ammonia is involved, since this compound has been convincingly shown to be an inducer of slug migration (Schindler & Sussman, 1977; see 1.3) but no direct evidence is available and other compounds have slug inducing activity eg. STF, Slug Turning Factor (Fisher et al., 1981; Fisher & Dohrmann, personal communication).

The role of ploidy in the slug-fruit switch is unknown. The increased sensitivity of DP82 compared to its haploid NP73 seems to be genotype dependent, since other haploid-diploid pairs did not show such a marked increase in sensitivity (data not shown).

Specific proteins are absent from the sheath of slugs migrated on cellulase containing agar (3.4.2). However, it is not possible to rule out that the cellulase mediated loss of sheath proteins occurs in the trails rather than the sheath proper. If this was so then the sheath actually surrounding the slug may contain its full complement of proteins. In addition, the same effect on sheath proteins is seen at 0.1mg/ml and 1.0mg.ml cellulase, so the effect is apparently maximal at concentrations well below those required for fruiting body induction. It is unlikely, therefore, that the sheath proteins detected by the mAb's play a direct role in the cellulase mediated induction of fruiting body formation.

This does not rule out some role for these proteins in the normal determination of sheath porosity. The phenotype of WS584 slugs provides some evidence for this. The sheath of these slugs is missing most of its cellulase-sensitive proteins and is similar in appearance and strength to the sheath from cellulase migrated slugs. In contrast to all of the other wild isolates examined, WS584 showed a marked tendency to form fruiting bodies rather than migratory slugs, possibly because of the lack of sheath proteins or the small size of its slugs (3.2.7).

(b) Cellulase, Diffusion and Cell Type

Proportions: Small diffusible molecules have been proposed to play a role in the establishment and/or maintenance of the correct proportions of cell types in the slug (see 1.3). Thus, if the cellulase induced slug-fruit switch is due to an increase in the rate of diffusion of a migration inducing molecule out of the slug, cellulase may also have caused changes in the cell type proportions in the slug. The absence of a cellulase effect on proportioning is not, however, inconsistent with a proportioning role for other small molecules which, for example, are active over short ranges (see Meinhardt, 1983) or are non-volatile (ie: where changes in diffusion out of the slug may be unimportant).

(c) The Sheath and Slug Migration: Slugs migrating on charcoal containing agar show changes in speed and orientation (see Appendix; Fisher & Williams, 1982). The effect on orientation is not related to the sheath (Fisher & Williams, 1982), but the effect on speed may be. The sheath from slugs migrating on charcoal containing agar is considerably weaker than from control slugs (which means, however, that it is impossible to collect and analyse).

In contrast to charcoal, neither slug speed nor orientation were detectably altered on cellulase containing agar (data not shown). If the sheath is involved in determining slug speed (Inouye & Takeuchi, 1979; see Appendix), the sheath proteins are likely to be the important component. However, cellulose may still play some part in slug migration because mutants which completely lack cellulose (Dimond et al., 1976) fail to migrate. The effect of this mutation on sheath proteins is unknown, although it

is clear that some protein must still be secreted (see 1.4).

In order to directly approach the question of the role of the sheath proteins an attempt was made to modify the phenotype of migrating slugs by including mAb's in the agar. No effect on speed, orientation or fruiting body formation was seen (data not shown). As a less specific probe for sheath protein function, slugs were treated with tunicamycin (Lehle & Tanner, 1976). In contrast to a previous report which claimed that the drug had no effect on slugs (Lam & Siu, 1982), slugs transferred to agar containing 5 μg/ml of tunicamycin ceased migration within a few hours and slugs at 15 μg/ml did not migrate at all. However, the drug affects a large number of proteins (Lam & Siu, 1982; Yamada et al., 1982) and results are impossible to interpret in terms of sheath involvement.

4.8 Conclusions and Prospects

The functional studies reported here are incomplete and inconclusive in many aspects but have provided evidence for a previously proposed role for the sheath as a barrier to diffusion in the slug-fruit switch. In addition, they provide some ideas as to what sort of phenotypes sheath mutants may display. For example, mutants with altered ShA may have weak sheath and stalk or an altered slug-fruit switch. Newell and Ross (1982) have reported that some slugger mutants (which migrate rather than form fruiting bodies) have sheath which is altered in appearence. Such mutants can now be screened for in a less haphazard way than previously. The polymorphism exhibited by WS584 may offer a starting point for a genetic study of the sheath should it

prove possible to correlate its slug phenotype (tendency to form fruiting bodies) with its lack of sheath proteins able to be detected by anti-sheath mAb's.

The antigenic and polymorphic differences between PsA and ShA will greatly facilitate the cloning and identification of their respective genes. This would allow the question of the relationship between them (and the 40kd protein) to be unambiguously resolved. It will also provide probes for the regulation of expression of the two proteins and solve the problem of how polymorphisms arose.

Finally, the work presented here demonstrates the tractability of the D.discoideum slime sheath as a model system for the study of extracellular matrices in a developmental context. The ability to perform relatively sophisticated genetic manipulations and the possbility that an easily used transformation system may soon be available in this organism (Hirth et al., 1982; Metz et al.,1983) are of enormous advantage in this respect.

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